BMP3b EXPRESSION AND ROLE IN ZONATION AND TUMOURIGENESIS IN THE ADRENAL CORTEX

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ABSTRACT

The adrenal gland is a vital organ consisting of an outer cortex surrounding an inner medulla. The adrenal cortex has three zones: zona glomerulosa (zG), zona fasciculata (zF) and zona reticularis (zR) which synthesise the steroids aldosterone, cortisol and dehydroepiandrosterone, respectively. The adrenal gland responds to constant physiological changes to maintain homeostasis by rapidly producing hormones, such as cortisol during stress or aldosterone in hypotension. The factors that determine differentiation and zonation of the adrenal cortex are not fully understood, although there is consensus that adrenal stem/progenitor cells residing in the subcapsular region of the gland can differentiate to renew and remodel the gland according to steroidogenic requirements.

Bone morphogenetic protein 3b (*Bmp3b*) was previously found in a rat microarray study to be exclusively expressed in the outer part of the adrenal cortex that contain capsular/zG cells. We have confirmed the findings of the microarray study and demonstrate that *BMP3b* acts locally to upregulate *CYP11B2* (specific to zG) and in a gradient dependent manner to downregulate the expression of steroidogenic enzymes specific to zF and zR in H295R cell lines. This action of *BMP3b* is possibly through inhibition of *BMP2* expression and activity. *BMP2* was found to be most highly expressed in the inner cortex and was shown to upregulate the expression of zR specific enzymes. Animal experiments confirmed the presence of *Bmp3b* in the capsular region but indicated that *Bmp3b* knockout mouse did not have abnormal adrenal morphology.

BMP3b expression was increased in some human adrenocortical carcinoma samples but this was not consistent across the cohort. Those tumours with increased expression of *BMP3b* correlated with a less aggressive histology. *BMP3b* could therefore potentially be used as a prognostic factor and help guide patient treatment and prognosis.

This study demonstrates that *BMP3b* could play a role, probably in concert with other factors, in adrenal zonation and tumourigenesis.

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ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase/isomerase
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
18-OHF	18-hydroxycortisol
β-TrCP	Beta-transducin repeats-containing protein
ActR	Activin receptor
ACAT	Acyl-CoA cholesterol transferase
ACC	Adrenocortical carcinoma
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AGP	Adrenogonadal primordium
ALK	Activin receptor-like kinase
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Adrenocortical primordium
APA	Aldosterone producing adenoma
APC	Adenomatous polyposis coli
AS	Aldosterone synthase
AT1	Angiotensin II receptor type 1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ATP1A1	ATPase Na^+/K^+ transporting subunit Alpha 1
ATP2B3	ATPase plasma membrane Ca^{2+} transporting 3
AVP	Arginine vasopressin
BAH	Bilateral adrenal hyperplasia
BCIP	5-bromo-4-chloro-3'-indolyphosphote p-toluidine salt
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	5-bromo-2'-deoxyuridine
CACNA1D	Calcium voltage-gated channel subunit Alpha 1 D
cAMP	Cyclic adenosine monophosphate
CaMK	Calmodulin dependent protein kinase
CBG	Corticosteroid binding globulin
cDNA	Complementary deoxyribonucleic acid
Cited2	CREB-binding protein/p300-interacting transactivator, with ED-rich tail, 2
CK1β	Casein kinase 1β
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator

CRH	Corticotrophin releasing hormone
CTNNB1	Catenin Beta 1
CYP11A1	Cytochrome P450 side chain cleavage/P450scc
CYP11B1	11β hydroxylase
CYP11B2	Aldosterone synthase
CYP17	Cytochrome P450 17α hydroxylase / 17,20 lyase
CYP21	21α hydroxylase
CytB5	Cytochrome B5
DAB	3,3'-diaminobenzidine
DAG	1,2-diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
Dax1	Dosage-sensitive sex-reversal adrenal hypoplasia congenital on the X chromosome, gene 1
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
Dhh	Desert hedgehog
DIG	Digoxigenin
DLK1	Delta-like 1 Homolog
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DOC	11-deoxycorticosterone
Dpp	Decapentaplegic
DPX	Distyrene, plasticiser and xylene mixture
DTT	Dithiothreitol
Dvl	Dishevelled
DZ	Definitive zone
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
Erk1/2	Extracellular-signal-regulated kinase 1/2
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
For	Forward
Fsk	Forskolin
FZ	Fetal zone
Fz	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF 10	Growth differentiation factor 10
GFP	Green fluorescent protein
GIRK4	G-Protein-Activated Inwardly Rectifying K^+ Channel 4
Gli	Glioma-associated

GliA	Gli activator
GliR	Gli repressor
GnRH	Gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
GRA	Glucocorticoid remedial aldosteronism
GRE	Glucocorticoid response element
GSK-3β	Glycogen synthase kinase-3β
H&E	Haematoxylin and Eosin
HDL	High density lipoprotein
Hh	Hedgehog
HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPA	Hypothalamic-pituitary-adrenal
HSL	Hormone sensitive lipase
Hsp90	Heat shock protein 90
hTERT	Human telomerase reverse transcriptase
I-smad	Inhibitory smad
IGF	Insulin–like growth factor
Ihh	Indian hedgehog
IHC	Immunohistochemistry
IMM	Inner mitochondrial membrane
IP_3	Inositol 1,4,5-triphosphate
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IZ	Inner zone
JNK	Jun N-terminal kinase
KCNJ5	Potassium channel, inwardly rectifying, subfamily J, member 5
KO	Knockout
LAL	Lysosomal acid lipase
LDL	Low density lipoprotein
LGR	Leucine-rich repeat-containing G-protein coupled receptor
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin 2 receptor
MMLV-RT	Maloney murine leukemia virus-reverse transcriptase
MR	Mineralocorticoid receptor
NAD^+	Nicotinamide adenine dinucleotide
NBRE	NGFI-B response element
NBT	Nitro-blue tetrazolium chloride
NGFI-B	Neuronal growth factor induced clone B
NOD	Non obese diabetic
NTC	No template control
MEN	Multiple endocrine neoplasia
miRNA	MicroRNA
mTOR	Mammalian target of rapamycin
OCT	Optimal cutting temperature compound
OD	Optical density
OGTT	Oral glucose tolerance test

PA	Primary aldosteronism
PBS	Phosphate buffered saline
PCC	Phaeochromocytoma
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
Pref1	Preadipocyte factor 1
Ptch1	Patched 1
qPCR	Quantitative polymerase chain reaction
R-smad	Receptor regulated smad
RAA	Renin angiotensin aldosterone
Rev	Reverse
RNA	Ribonucleic acid
RSPO3	R-spondin-3
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SAG	Smoothened agonist
SF-1	Steroidogenic factor 1
Sfrps	Frizzled related proteins
Shh	Sonic hedgehog
siRNA	Small interfering RNA
Sgk1	Serum/glucocorticoid regulated kinase 1
SNS	Sympathetic nervous system
Smo	Smoothened
Smurf 1	Smad ubiquitin regulatory factor 1
SOC	Super optimal broth with catabolite repression
SR-B1	Scavenger receptor class B type 1
SSC	Sodium saline citrate
StAR	Steroidogenic acute regulatory protein
SuFu	Suppressor of fused
SULT2A1	Sulphonyltransferase family 2A member 1
TAE	Tris base, acetic acid and EDTA
Taq	*Thermus aquaticus*
Tcf/Lef	T cell-factor/lymphoid enhancer factor
TESPA	3-triethoxysilylpropylamine
TGF-β	Transforming Growth Factor β
TH	Tyrosine hydroxylase
Tm	Melting temperature
TMB	Tetramethylybenzidine
Tsg	Twisted gastrulation
TZ	Transitional zone
Un	Untreated
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related mouse mammary tumour virus integration site
WT	Wild type

WT1	Wilm's tumour 1
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
zF	Zona Fasciculata
zG	Zona Glomerulosa
zR	Zona Reticularis
zU	Undifferentiated zone

1 Introduction

1.1 The stress system: Hypothalamo-pituitary-adrenal (HPA) Axis

Hans Selye was the first to describe a biological response to stress as a general adaptation syndrome in 1936 (Selye, 1936). Stress is defined as a real or anticipated disruption of homeostasis, or an anticipated threat to well-being (Ulrich-Lai and Herman, 2009). This can be an emotional or physiological response to an external/environmental situation. For example, it can be a non-specific response of the body to any demand placed upon it or a life-threatening situation such as a major physical trauma, prolonged starvation or illness. Stress has also been summarised as any condition where an environmental demand exceeds the natural regulatory capacity of an organism (Koolhaas et al., 2011). Selye observed the behavioural, hormonal, and autonomic responses to stress. These changes occur as a result of the activation of the autonomic nervous system (ANS) as the immediate response which is followed by activation of the hypothalamic-pituitary-adrenal axis (HPA). In response to stress, both the sympathetic and parasympathetic systems lead to immediate changes in the body's physiological state through the action of the adrenal medulla and secretion of catecholamines (adrenaline and noradrenaline), termed the 'fight or flight' response. This response is rapid and also wanes quickly as the parasympathetic system activates. If stress is prolonged then the HPA is activated and cortisol is produced, with an approximate delay of 10 minutes from the onset of stress (Droste et al., 2008). Upon activation of the HPA axis, the paraventricular nucleus synthesises and secretes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus into the hypophyseal portal circulation (Carrasco and Van De Kar, 2003; Chrousos, 1995; Sapolsky et al., 2000). Stress is also associated with the release of other hormones such as oxytocin, prolactin and renin in order to ensure maximum ability for survival, should there be dysregulation in the main stress HPA axis (Aguilera, et al., 1995; Carrasco and Van De Kar, 2003; Levine and Muneyyirci-Delale, 2018; Onaka and Takayanagi, 2019). CRH, along with AVP, works synergistically acting on the corticotroph cells of the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the systemic circulation (Chrousos, 1995; DeBold et al., 1984). ACTH is a 39 amino acid peptide derived from the cleavage of proopiomelanocortin (POMC) by the peptidases PC1/PC3 (Chan

et al., 2011). The release of CRH, AVP and ACTH happen in a matter of seconds (Sapolsky et al., 2000).

ACTH released from the anterior pituitary into the peripheral circulation acts on the ACTH receptor, also known as the melanocortin 2 receptor on the surface of adrenal cells to increase the production of glucocorticoids (cortisol in humans and corticosterone in rodents). The presence of glucocorticoid will then act on the hypothalamus and pituitary to negatively feedback and supress further release of CRH and ACTH.

The HPA axis is constantly regulated to maintain homeostasis. Without stress, circulating ACTH and glucocorticoid levels fluctuate in a pulsatile, circadian manner over a 24 hour period (Carnes et al., 1988; Spiga et al., 2011; Windle et al., 1998). In humans, cortisol levels peak between the hours of 0600-0900 am, on waking. Cortisol levels then fall throughout the day, reaching a nadir at midnight, after which levels slowly rise again. On waking, cortisol is required to increase energy level, increase blood pressure and activate gluconeogenesis. Cortisol levels are very low by night time in preparation for sleep. This pulsatility is vital as chronic exposure to glucocorticoids leads to desensitisation of the body's response to stress (Spiga et al., 2011). Over time, if the stressor prevails, our body can become conditioned, resulting in a blunted response to stress as the system is desensitised. This chronic stress can also lead to disease, such as hypertension, diabetes and gastric ulcers, as well as affecting growth and fertility (Sapolsky et al., 2000).

More recently, the 24 hour circadian rhythm of glucocorticoids have been further investigated and it is now clear that the nature of pulses is far more dynamic, consisting of oscillatory pulses of ACTH and glucocorticoids known as the ultradian rhythm. The ultradian rhythm consists of regular pulses of ACTH which then stimulate the adrenal gland to produce glucocorticoids, with a built in delay at the adrenal cortex. The glucocorticoids then feed back to the pituitary to suppress any further ACTH release. This system of regular pulsitility is thought to produce a self-sustaining oscillatory activity of glucocorticoid ultradian rhythm (Russell and Lightman, 2019; Walker et al., 2010).

1.2 The adrenal gland

The adrenal gland is an important organ that is essential for life. It is the key organ for the regulation of the stress response, producing hormones that maintain blood pressure and glucose utilisation at times of illness and injury. The adrenal gland has three distinct outer zones, which are responsible for the production of steroid hormones. The organisation and zonation of the gland is critical for the normal function and production of these hormones. It is thought that all three zones of the adrenal cortex derive from a single source of progenitor cells. Understanding the process of zonation and steroidogenesis will provide insight into conditions where there is over and underproduction of hormones from the adrenal gland and adrenal carcinomas, where survival remains poor.

1.2.1 Development of the adrenal glands

The adrenal medulla has a different developmental origin to the adrenal cortex with the former arising from the neuroectoderm, where cells of the neural crest lineage migrate in to the adrenal anlage ultimately forming the chromaffin cells of the medulla (Mesiano and Jaffe, 1997). The medulla is part of the sympathetic nervous system and is responsible for the secretion of catecholamines (Carrasco and Van De Kar, 2003; Chrousos, 1995; Miller and O'Callaghan, 2002).

The adrenal cortex in contrast originates from the urogenital ridge, as do the gonads and the kidneys. Human adrenal development begins around the fourth week of gestation from the mesoderm, where the coelomic epithelium thickens and the underlying mesonephric mesenchymal cells, found in the notch between the primitive urogenital ridge and the dorsal mesentery, migrate to form the adrenogonadal primordium and the kidney (Hatano et al., 1996; Ikeda et al., 1994; Parker et al., 2002). Development and division of the adrenogonadal primordia (AGP) occur by week 8, when there is a distinct separation into the adrenocortical and gonadal primordium. This then becomes the fetal adrenal (see Figure 1.1) that comprises of a large inner fetal zone (FZ) composed of large eosinophilic cells that are capable of steroidogenesis. The FZ is surrounded by a very thin outer layer of tightly packed basophilic

cells known as the definitive zone (DZ) that have no steroidogenic role during development. In between the two zones lies the transitional zone (TZ). The transitional zone is also able to synthesise cortisol after mid-gestation. By the 30th week of gestation, the DZ and TZ begin to resemble zG and zF of the adult adrenal cortex respectively (Mesiano et al., 1993). At birth, the FZ begins to regress by apoptosis and the DZ and TZ begin to expand to form the zG and zF. By age 3 years the zG and zF are fully formed (Spencer et al., 1995, 1999). In primates, the zR begins to form producing androgens after the age of 6, a process known as adrenarche, and may continue to grow until the age of fifteen (Kempná and Flück, 2008; Kim and Hammer, 2007).

During development, the transcription factor Sf-1 (steroidogenic factor 1) is the earliest marker of steroidogenic cells in the adrenogonadal primordium and it is essential for the development of both the adrenals and the gonads (Kim and Hammer, 2007; Mesiano and Jaffe, 1997).

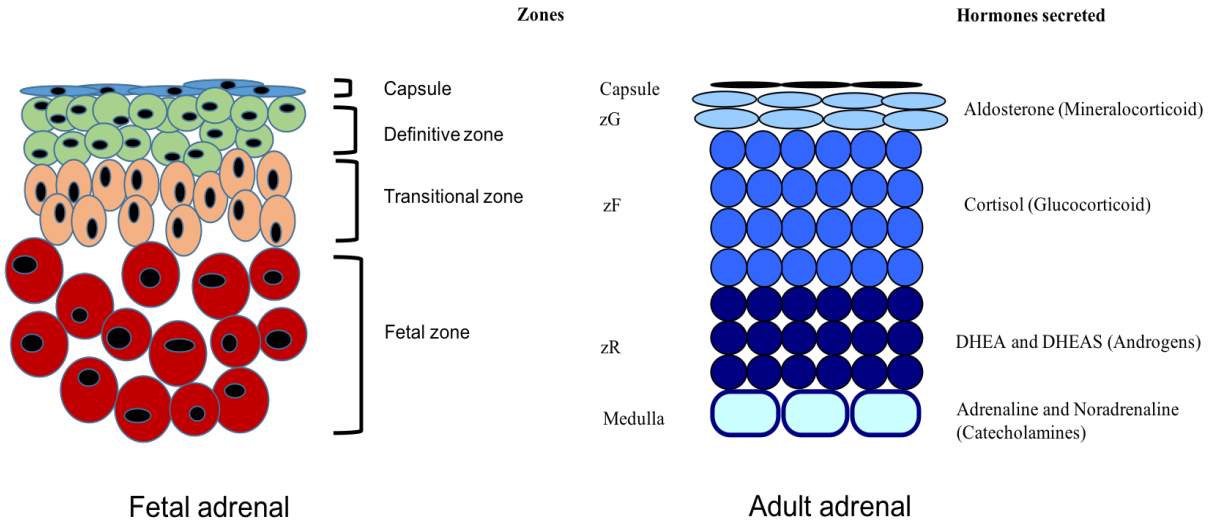

Figure 1.1 Adrenocortical zones in the human adrenal gland in fetal development and adult with its associated hormone production
The fetal adrenal consists of the inner fetal zone containing large eosinophilic cells in the centre with the definitive zone subcapsularly and the transition zone lying in the middle. The adult adrenal cortex consists of 3 concentric zones: Outer zG secretes aldosterone, inner zF secretes glucocorticoids and innermost zR secretes DHEA and DHEA-S. The cortex abuts the adrenal medulla which secretes adrenaline and noradrenaline. The gland is encapsulated by the adrenal capsule. zG = zona Glomerulosa, zF = zona Fasciculata and zR = zona Reticularis, DHEAS = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulphate.

In the mouse, the adrenogonadal primordium is first detected at embryonic day 9.5 (E9.5) with expression of *Sf-1 and Dax-1*. Development and division of the adrenogonadal primordia (AGP) into the gonadal and adrenal primordia occurs by E10.5. The zF forms by E14.5, but the zG is not observed until the perinatal stages of E20 when expression of *Cyp11b2* is present (King et al., 2009; Mitani et al., 1999). Mice have an equivalent zone to the human FZ, the X-zone, which regresses in male mice at puberty (about 5 weeks of age) and in females after their first pregnancy (Holmes and Dickson, 1971). Adrenal development in rats is similar to mice but with developmental milestones that are typically delayed by one day. Rats do not have an equivalent X zone (Klepac et al., 1977; Mitani et al., 1999).

Apart from histological differences, another important difference between the primate and rodent adrenal gland is the lack of a functional zR in rodents. This is due to a lack of CYP17 enzyme, necessary for the androgen synthesis. In rats, there is the formation of a fourth zone; the undifferentiated zone (zU) that does not stain for any steroidogenic antibodies (CYP11B1, CYP11B2 and CYP17). This zone is not seen in many other species, such as mice, pig or rabbit and its size is also variable across rat strains, being most prominent in Sprague-Dawley rats (Mitani, 2014; Xing et al., 2015).

1.2.2 Adrenal anatomy

The adrenal gland is a component of the HPA axis, producing an array of hormones that are essential for life, which, if dysfunctional, is associated with mortality and morbidity (Kempná and Flück, 2008). The first description of the adrenal gland was made in the second century by the Greek physician, Claudius Galenus of Pergamon (Galen), but the first definitive illustration of the gland was by Bartolomeo Eustachio (an Italian anatomist) in the 16th century in his book entitled 'Tabular Anatomica. Chapter VL, Vemica' (Leoutsakos and Leoutsakos, 2008; Mezzogiorno and Mezzogiorno, 1999; Rochester, 1989). In the 18th century, Cuvier Lenard first used the terms 'cortical' and 'medulla' and recognised that they were distinct morphological entities within the adrenal gland (Lenard, 1951). However, it was not until 1852 when significant advances in microscopy allowed Albert Von Kölliker to fully describe the cortex and medulla as two physiologically and functionally distinct areas within the adrenal

gland (Leoutsakos and Leoutsakos, 2008). In 1855, Thomas Addison was the first to recognise the importance of the adrenal gland and that it was essential for life. In his monograph, 'On the constitutional and local effects of disease of the supra renal capsules', he described patients with destruction of the adrenal gland (due to tuberculosis) to have "anemia, debility, feebleness of the heart, irritability of the stomach and peculiar changes of the color in the skin, occurring in connection with a diseased condition of the suprarenal capsules". The condition was later named "Addison's disease" (Pearce, 2004). Charles-Edouard Brown-Sequard found that animals could not survive if bilateral adrenalectomy was performed (Brown-Sequard, 1856; Miller, 2013), thus demonstrating that the glands are essential for life. Ever since its discovery, there has been a vast amount of work investigating the physiology, development and maintenance of this gland in health and disease.

The adrenal glands are V, or pyramidal shaped on the right side and Y, or crescent shaped on the left. In adult humans they weigh approximately 8-10 g each and in rats 20-30mg each. They sit anterosuperiorly to the kidneys. Each adrenal gland is contained and surrounded by a capsule, made up of fibroblasts and myofibroblasts. A network of arterioles lie subcapularly, forming a plexus that supplies blood to the adrenal cortex and medulla (Ishimoto and Jaffe, 2011; Mesiano and Jaffe, 1997).

Beneath the adrenal capsule is the adrenal cortex (cortex, meaning 'bark'), which accounts for approximately 90% of the adrenal mass in healthy individuals. The cortex is divided into three histologically and functionally distinct concentric zones: the outermost zona glomerulosa (zG), the middle zona fasciculata (zF) and the innermost zona reticularis (zR) (Figure 1.1) (Mitani, 2014; Yates et al., 2013). The zG is named due to its appearance being similar to the glomeruli of the kidneys, with cells arranged in 'balls' (Latin glomus = ball). The zG is a narrow zone with small columnar cells arranged in round clusters with intense nuclei and basophilic cytoplasm with a low lipid content. It produces the mineralocorticoid aldosterone, which is part of the feedback system of the renin-angiotensin adrenal system to regulate sodium balance via the kidneys, maintaining water and blood pressure homeostasis (discussed in more detail in Section 1.5). The zF, named from the appearance of cells lining up in parallel fascicles or bundles, is responsible for the production of cortisol in humans and corticosterone in rodents.

Glucocorticoids are known as the stress hormone because at times of stress, an increased level of cortisol is produced and this allows the body to upregulate mechanisms to deal with the stress, such as gluconeogenesis. Glucocorticoids are essential in maintaining homeostasis during stressful events by controlling metabolism and affecting vascular tone. This includes the regulation of glucose by glucocorticoids, in order to maintain glucose supply for maximal brain function at times of stress. It does this by promoting gluconeogenesis in the liver and increasing insulin resistance in muscles and white adipose tissue (Kuo et al., 2015) by inhibition of GLUT4 recruitment (Weinstein et al., 1998). Glucocorticoids also have a role in glycogen storage in the liver and glycogen usage in skeletal muscle as well as the secretion of glucagon and insulin through pancreatic α and β cells (Kuo et al., 2015).

The innermost zone of the cortex, which is found in humans and primates but not rodents, is the zR. Histologically, the cells appear as a network or "cord" of cells hence giving it its name (Latin rete = network). The zR is responsible for the production of adrenal androgens, and is involved in sexual development (Rainey, 1999; Vinson, 2016).

The most inner part of the adrenal gland is the medulla (meaning 'marrow' or core) which has a separate embryological origin and function to the cortex. It produces catecholamines (adrenaline and noradrenaline), as part of the fight and flight hormones and contributes to the sympathetic nervous system (Kempná and Flück, 2008).

1.3 Zonation theories

Research into adrenal development and steroidogenesis has provided insight into the signalling pathways and the function of the adrenal cortex. Zonation research has expanded over the last two decades with many pathways implicated in the formation of the various zones within the adrenal cortex as well as its maintenance.

The adrenal gland is a highly dynamic organ with the capability to remodel rapidly in response to homeostatic perturbations (Dallman et al., 1976; Kawai et al., 1979; Kifor et al., 1991). Rats

fed on a low sodium diet will exhibit an expansion of the zG (Cohen, 1965; Mitani et al., 1994)due to activation of the RAAS (Renin Angiotensin Aldosterone System) to conserve sodium. This results in increased AT1 and AT2 mRNA levels, to allow for increased receptor expression and Ang II binding (Aguilera et al., 1980). Increased levels of Ang II will result in increased production of aldosterone in the zG to act on the collecting tubules in the kidneys to increase sodium reabsorption (Pacha et al., 1993). To accommodate the high levels of aldosterone production, the zG layer expands as a result.

ACTH administration causes the zF to expand. ACTH exerts its effects via cAMP, resulting in upregulation of ACTH receptors (de Joussineau et al., 2012). This leads to an increase in the synthesis of enzymes within the steroidogenesis pathway necessary for increased glucocorticoid production (Pudney et al., 1984; Simpson and Waterman, 1988). Unilateral adrenalectomy will cause the remaining contralateral adrenal to compensate and become hyperplastic (Engeland et al., 2005; Sidorova and Stepanova, 1983). In earlier studies, this hyperplasia was thought to occur in the capsule, zG and outer zF (Holzwarth et al., 1996). However, more recent studies by Engeland *et al.,* using triple label immunostaining against aldosterone synthase to label the zG, 11β-hydroxylase to label the zF and a mouse monoclonal antibody against Ki-67. Ki-67 is an antigen present in all active phases of the cell cycle and in this study demonstrated that cell proliferation occurred mainly in the outer zF (Engeland et al., 2005).

The adrenal cortex is rapidly responsive to stresses the body is subjected to. In order for the adrenal glands to respond by increasing aldosterone or cortisol secretion as necessary, it is thought that a pool of undifferentiated, pluripotent cells exist, ready to be differentiated as required, in order to maintain homeostasis. The idea of possible progenitor cells stemmed from studies in the 1950s and 1960s demonstrating regeneration in zonation and function of the adrenal gland following bilateral adrenal enucleation in rats (Mesiano et al., 1993; Skelton, 1959). For adrenal enucleation, the rats were anaesthetised and an incision was made through the flank, exposing the adrenal glands. A slit was made in the adrenal capsule and the inner regions of the gland were squeezed out using small forceps (Alexander et al., 1976) leaving only the capsule and adherent cells. The cortex re-established itself with appropriate zonation

within a few weeks (Engeland and Levay-Young, 1999; Engeland et al., 1995) indicating that pluripotent cells were present in the capsule or subcapsular region that can differentiate into both zG and zF cells. More recent studies also support this idea (Ennen et al., 2005; Perrone et al., 1986). These data, together with early lineage tracing experiments using trypan blue (Salmon and Zwemer, 1941) or tritiated thymidine (Wright et al., 1973) led to the idea that progenitors at the capsule/subcapsular region migrate towards the medulla in a centripetal manner. During this migration the cells differentiate into the properties of the zone in which they are traversing and undergo apoptosis at the medulla boundary. This is currently the most accepted theory of zonation. Regeneration experiments by Kim *et al*., took donor capsule/subcapsular units and implanted them subcutaneously into syngeneic mice. They observed the regrowth of *Sf-1* positive cells and by 3 months, the entire cortex was repopulated and functioning (Kim et al., 2009). In other studies where primary adrenocortical cells were implanted into immunodeficient mice, steroidogenic potential was evident but no zonation or continued cell renewal was seen (Thomas and Hornsby, 1999; Thomas et al., 2002), highlighting the importance of the presence of capsular/subcapsular tissue for regeneration.

Further confirmation supporting the centripetal migration theory was carried out by King *et al.,* using both *Shh-cre* and importantly *Gli1-cre*, to specifically follow the capsule cells during development. They found that these cells populated the entire cortex, at least in part via an *Shh* expressing intermediate (King et al., 2009). Freedman *et al*., followed on from this to show direct conversion of *Cyp11b2* to *Cyp11b1* cells, by introducing Cre recombinase into the genetic locus for *Cyp11b2* (aldosterone synthase). *AS-Cre+/-* mice were then crossed with a R26R+/mTmG mice. Using this mouse model, the zG cells were GFP positive at birth in the subcapsular region and by 12 weeks of age, zF cells were noted to be GFP positive, indicating that they were descended from the once *Cyp11b2* expressing zG cells. The direct lineage conversion was suppressed by treatment with dexamethasone. Furthermore, zG maintenance and lineage conversion was also shown to be dependent on *Sf-1* and zG cells were unable to convert to zF cells in the absence of *Sf-1*. However, despite that, a fully functional zF is maintained throughout life in these mice, indicating that a zG independent mechanism is also present to make and maintain zF cells (Freedman et al., 2013).

Alternative theories on the location of progenitor cells that have fallen out of favour include the zonal hypothesis and the transformation field hypothesis. The zonal hypothesis dictates that each zone will have their own pool of cells that will increase or decrease under the control of its own zone. The transformation field hypothesis predicted that cells resided on the zG/zF border and the zF/zR border and cells would be transformed as needed and regress when they were not required (Vinson, 2003; Wolkersdorfer and Bornstein, 1998). These alternative theories may be out of favour following the studies performed by King *et al.,* and Freedman *et al.,* as described above and suggesting that more than one mechanism for progenitor cell maintenance and differentiation exists (Freedman et al., 2013; King et al., 2009).

The localisation of the adrenal progenitor cells may differ between species. Mitani *et al.,* defined the physiological significance of a zone in the rat adrenal that is 5-10 cell layers thick under normal conditions, that lies in between the zG and zF. This zone is known as the undifferentiated zone (zU) (Mitani et al., 1994). The zU was first described in the 1930s, but Mitani's group were the first to discover that cells in the zU do not express the zF or zG terminal steroidogenic enzymes (Cyp11b1 and Cyp11b2 respectively) and do not produce adrenal androgens due to a lack of CYP17 enzymes, indicating that they are non-steroidogenic. They proposed that the progenitor cells resided within this layer because these cells do express *Sf-1*, indicating steroidogenic capacity. This is further supported by their BrdU studies, where BrdU was incorporated preferentially into cells around the zU, which had a high mitotic rate. By subjecting rats to a salt deficient diet, they were able to show an increase in BrdU-positive cells within the outer zU that abut the zG. This is in contrast to normal conditions, where BrdU-positive cells would reside nearer the inner zU that abuts the zF (Mitani et al., 1994). The location of the adrenal progenitor cells within the undifferentiated zone of the rat adrenal gland is further supported by work by Guasti *et al.,*. In this study, *Shh* was found to be expressed in the outer half of the zU in rat adrenal (Guasti et al., 2010). Ablation of *Shh* in adrenal specific manner was achieved by crossing *Shh* floxed mice with a *Sf-1*-Cre mice. Analysis of the adrenal glands from *Shh* conditional KO animals demonstrated a reduction in proliferation of capsular cells, reduction in cortical thickness and smaller adrenal glands. These mice, despite having morphologically smaller adrenal glands, were still capable of undergoing zonation and producing steroids, indicating that SHH is more likely to have a role in stem/progenitor cell

dependent cell maintenance than zonation (Huang et al., 2010). Taken together, these data suggest that the *Shh* expressing cells could represent the stem/progenitor population in adult animals and respond to changes in homeostasis by differentiating into zG or zF cells as required (Guasti et al., 2010).

Whether stem /progenitor cells reside subcapsularly or in the undifferentiated zone or both remains unclear. However, there are likely to be species differences such as in rats and mice. For example, SHH positive cells reside in the zU in rats whilst such cells cluster beneath the capsule in mice (King et al., 2009), with no apparent zU of note. This could explain the differences in zonation theories that have been suggested through the years, although there is agreement that the stem/progenitor cell niche exists in the adrenal gland, and these are non-steroidogenic with the potential to become steroidogenic cells to maintain homeostasis. There are now a growing number of genes and pathways that have been implicated in stem/progenitor cell function that determine adrenal gland development, zonation and differentiation (El Wakil and Lalli, 2011; Guasti et al., 2013a; King et al., 2009; Wolkersdorfer and Bornstein, 1998).

1.4 Adrenal function and Steroidogenesis

All steroids are synthesised from the precursor cholesterol. Cholesterol is obtained from exogenous dietary sources and transported through the circulation in lipoprotein particles, principally from low density lipoproteins (LDL) (Gwynne and Strauss, 1982) via LDL-receptor mediated endocytic pathway (Goldstein and Brown, 2009) and a lesser contribution from high density lipoproteins (HDL) via SR-B1/selective pathway (Azhar and Reaven, 2002; Azhar et al., 2003). Stored cholesterol, in the form of cholesterol esters in lipid droplets, can be released for steroidogenesis upon unesterification by lysosomal acid lipase (LAL) (Du et al., 1998; Goldstein et al., 1975). Cholesterol can also be synthesised de novo from acetate (Mason and Rainey, 1987). The availability of cholesterol is dependent on the presence of ACTH, angiotensin II and potassium, where increasing levels of these agents would increase the expression and activity of LDL receptors, stimulating HMG CoA reductase and hormone sensitive lipase (Lehoux and Lefebvre, 1991).

Cholesterol is transported to the mitochondria by a number of mechanisms such as vesicular trafficking, by interaction with sterol carrier proteins or cytoskeleton proteins and by specific plasma membrane-mitochondrial or ER-mitochondrial interactions (Ikonen, 2008). Cholesterol is then trafficked from the outer to the inner mitochondrial membrane (IMM) by the transporter steroidogenic acute regulatory protein (StAR). This is the rate limiting step in steroid synthesis (Jefcoate, 2002; Simpson and Waterman, 1983) regulated by ACTH, which can rapidly stimulate production and activation of StAR (Arakane et al., 1997). Once inside the IMM, steroid synthesis begins through a multi-enzymatic process (Figure 1.2). The first step is the conversion of cholesterol to pregnenolone by cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1 or P450scc). From this point on, all the other steroid hormones can be synthesised, dependent on the isoform of 'cytochrome P450' present. Pregnenolone can then be converted into progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD, HSD3B) or transported to the smooth endoplasmic reticulum, where some of the enzymatic processes take place, P450c17 (CYP17A1) catalyses 17α-hydroxylase and 17,20 lyase. Following this, 21-hydroxylation occurs with P450c21 (CYP21A1) before the final steps of steroid synthesis occur, back in the mitochondria. Within the mitochondria, 2 isoenzymes of P450c11 are present for the final steps of steroidogenesis, these are aldosterone synthase (CYP11B2) and 11β–hydroxylase (CYP11B1). CYP11B2 is found exclusively in the zG for the production of aldosterone by the catalysis of 11β-hydroxylase, 18-hydroxylase and 18-methyl oxidase to convert deoxycorticosterone to aldosterone. Similarly, CYP11B1 is exclusive to the zF for the production of cortisol after 11β-hydroxylation of 11-deoxycortisol (Payne and Hales, 2004). Androgen production require the same initial steps to produce dehydroepiandrosterone (DHEA), relying on both CYP17 isoenzymes (Rainey et al., 1994, 2004; Staels et al., 1993) with the process being regulated by cytochrome b5 (CYTB5) to stimulate 17,20 lyase activity in the zR (Akhtar et al., 2005; Auchus et al., 1998). DHEA-sulfotransferase (SULT2A1) is another important enzyme within the zR as it can use pregnenolone, 17α hydroxypregnenolone and DHEA as its substrate to form DHEA-S (Rainey and Nakamura, 2008).

Steroidogenesis differs in rodents due to the lack of enzyme CYP17 (cytochrome P450 c17), which is required for both cortisol and androgen production via its 17α-hydroxylase and 17,20 lyase activities respectively. Without CYP17, a hydroxyl (-OH) group cannot be added to the C17 position (by 17α-hydroxylase) to progesterone or pregnenolone to form 17α-hydroxyprogesterone and 17α-hydroxyprenenolone, respectively. 17,20 lyase would then cleave the C17-C20 bond of either 17α-hydroxyprogesterone and 17α-hydroxyprenenolone to form androstenedione and DHEA respectively (Akhtar et al., 2005). Therefore, in the absence of CYP17, these substrates would not be available for rodents to produce cortisol or androgens. Hence rodents are only capable of producing corticosterone and aldosterone.

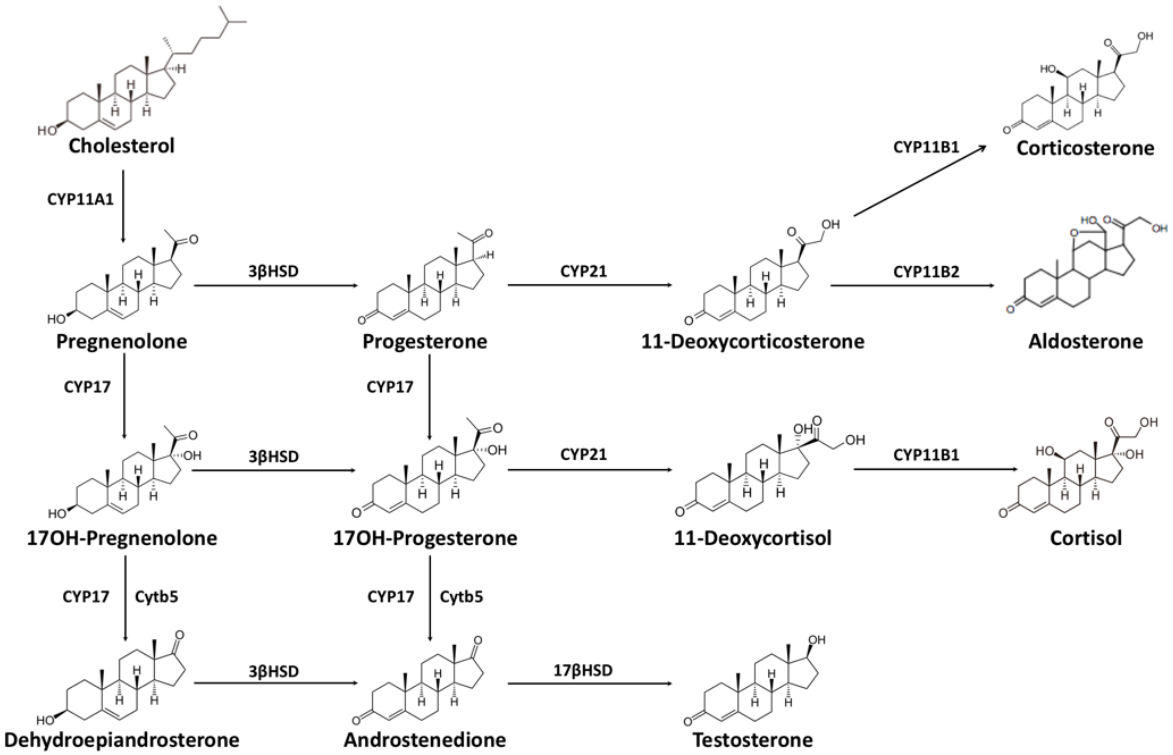

Figure 1.2 Biosynthesis of human steroid hormones
Multiple enzymatic steps necessary for the conversion of cholesterol to steroid hormones. Cholesterol is the common precursor for all steroid production and the conversion to pregnenolone by CYP11A1 (P450scc) is the rate limiting step and is present in all 3 zones. Prenenolone is trafficked back to the mitochondria. Pregnenolone then undergoes 17α-hydroxylation or 17,20 lyase to convert 17OH-pregnenolone. 17OH-pregnenolone, progesterone and 17OH-progesterone can then continue towards androgen production (dehydroepiandrosterone and androstenedione) if co-factors, such as cytochrome b5 in the reticularis is present to favour 17,20 lyase activity. Pregnenolone, 17OH-pregnenolone and dehydroepiandrosterone can also undergo conversion of their C-3 hydroxyl to a keto group to form progesterone, 17OH-progesterone and androstenedione. 17βHSD is responsible for the conversion of androstenedione to testosterone, which can then interconvert to oestrogen. CYP21 is a 21α-hydroxylase enzyme and converts progesterone to 11-deoxycorticosterone and 17OH-progesterone to 11-deoxycortisol. 11-deoxycorticosterone and 11-deoxycortisol are trafficked back into the IMM, where the final step takes place. 11-deoxycortisol is converted to cortisol through 11-hydroxylation by CYP11B1 and 11-deoxycorticosterone is converted to aldosterone by CYP11B2 (P450c11AS), catalyzing 18-hydroxylation and 18-methly-oxidation.

1.4.1 Cortisol, aldosterone and adrenal androgens

Cortisol is a glucocorticoid produced in the zF of the adrenal cortex. The role of glucocorticoid includes metabolic, cardiovascular and anti-inflammatory responses. About 90% of the cortisol produced is bound to corticosteroid binding globulins (CBG) in the blood and is biologically inactive (Breuner and Orchinik, 2002). The unbound cortisol, known as free cortisol, is the biologically active form. There are two isoforms of 11β-hydroxysteroid dehydrogenase (type 1 and type 2), responsible for the interconversion of the active form, cortisol to the inactive form, cortisone and vice versa. 11β-HSD1 acts as a reductase, allowing cortisol action in target tissues. 11β-HSD2 is a NAD-dependent dehydrogenase which converts cortisol into cortisone to prevent, for example, cortisol from acting on mineralocorticoid receptors in the kidney distal tubule (see below) (Stewart and Krozowski, 1999; Tannin et al., 1991; Yang and Zhang, 2004). CBG and cortisol will dissociate upon stress and this is believed to occur due to the release of elastase from activated neutrophils (Beishuizen et al., 2001; Hammond et al., 1990; Pemberton et al., 1988).

The action of cortisol is exerted on binding to glucocorticoid receptors (GR) in the cytoplasm of target cells. GR is a member of the nuclear receptor family of ligand-dependent transcription factors. Upon cortisol binding, the GR dissociates from a large complex of heat shock proteins and FK506-binding proteins (Lai et al., 2003; Pratt and Toft, 1997; Sinars et al., 2003). The GR is then phosphorylated upon ligand binding and translocate to the nucleus and binds to glucocorticoid-responsive elements (GREs) in the genome (Tsai et al., 1988; Wrange et al., 1989). This results in the recruitment of coactivator or corepressor proteins to either up- or down-regulate target genes under the control of GREs. The cortisol-GR complex also has the ability to modulate transcription factors in other pathways, such as the inflammatory and immune pathways (De Bosscher et al., 2003; Rhen and Cidlowski, 2005). Glucocorticoid receptors can also exert non genomic effects where they can rapidly regulate other signalling pathways, such as the mitogen-activated protein kinase (MAPK) and P13K/Akt signalling pathway (Ayroldi et al., 2012; Hafezi-Moghadam et al., 2002; Kharwanlang and Sharma, 2011).

Aldosterone is the mineralocorticoid secreted by the zG of the adrenal cortex. Its role is in controlling electrolyte and water balance via the kidneys to maintain optimal blood pressure. However, aldosterone does not act exclusively in the kidneys. It also acts on the CNS and the cardiovascular system to maintain blood pressure (Cannavo et al., 2018; Connell and Davies, 2005; Gomez-Sanchez, 1997; Huang and Leenen, 2011; Ren et al., 2019). The main control of aldosterone is by angiotensin II (Ang II) and potassium (K^+) and to a lesser extent ACTH and Vasopressin (VP) (El Ghorayeb et al., 2016; Muller, 1987; Quinn and Williams, 1988) but many other factors have been implicated, such as ouabain, a toxin derived from Acokanthera schimperi and Strophanthus plants (Huang and Leenen, 2011). Aldosterone production can be inhibited by dopamine and atrial natriuretic peptide (ANP) (Cannavo et al., 2018; Kohzuki et al., 1989; Mc Kenna et al., 1979).

Ang II stimulates aldosterone production in the zG via the angiotensin 1 receptor (AT1), which is a GCPR coupled to $G_{\alpha q}$. On stimulation of the AT1 receptors, phospholipase C is released to stimulate protein kinase C (PKC) activation by the increase in the production of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). A rise in IP_3 increases intracellular free calcium which in turn leads to the phosphorylation and activation of transcription factors such as activating transcription factors 1 and 2 (ATF), and cAMP response element binding protein (CREB) via calcium/calmodulin-dependent protein kinases (Hunyady and Catt, 2006). This results, within minutes, in binding of the transcription factors to cis-acting elements in the promoter of the *CYP11B2* gene. Ang II and K^+ increases the synthesis of neuronal growth factor induced clone-B (NGFI-B) to upregulate *CYP11B2* expression. Furthermore, Ang II and K^+ work synergistically with ATF/CREB to increase *CYP11B2* expression. CRE and NBRE (NGFI-B response element) are important in aldosterone production as mutations in these response element regions result in a 70% and 40% reduction in *CYP11B2* promoter responsiveness, respectively. Mutations in both CRE and NBRE result in the complete inability of AngII or K^+ to induce expression of *CYP11B2* (Nogueira and Rainey, 2010; Nogueira et al., 2009). Chronic Ang II stimulation leads to hypertrophy and hyperplasia of the zG with increased production of aldosterone via increased *CYP11B2* gene expression.

Aldosterone production is also stimulated by increased extracellular potassium concentration, independent of the RAAS. This is achieved by cell membrane depolarization and activation of voltage-dependent L- and T- type Ca^{2+} channels, resulting in a rise in extracellular calcium levels. This in turn activates calmodulin dependent kinase resulting in activation of transcription factors such as CREB to promote CYP11B2 gene expression (Nanba et al., 2015; Spat and Hunyady, 2004; Spät et al., 2016). ACTH exerts its main effects on glucocorticoid production in the zF but does have an effect on aldosterone production. Acutely, ACTH increases aldosterone production via cAMP pathways (Cozza et al., 1990) but chronically, it appears to suppress aldosterone levels (Aguilera et al., 1996). Further evidence is that ACTH receptor knockout mice are aldosterone insufficient, although this is not seen in patients with mutations in the ACTH receptor (Chida et al., 2009; Novoselova et al., 2019).

The action of aldosterone is initiated upon binding to mineralocorticoid receptors (MR) which, like GRs, are steroid receptors located in the cell cytosol. In the cytosol, unliganded MR is part of a multiprotein complex, including heat shock protein 90 (Hsp90) and immunophilins (Faresse et al., 2010). Aldosterone binding to MR leads to dissociation of the MR to enable translocation of the receptor to the nucleus where it homodimerises and acts as a transcription factor (Couette et al., 1998). Within the nucleus, the complex binds to steroid responsive elements in the promoter leading to an upregulation or downregulation of aldosterone responsive target genes. The complex can also act indirectly via other transcription factors to bind target DNA through a process known as transcription interference or synergy (Karin, 1998). Sgk1 (serum/glucocorticoid regulated kinase isoform 1) is a serine threonine kinase that activates sodium channels together with ENaC (epithelial sodium channel) to promote resorption of sodium in the distal renal tubules and is a well-established example of an aldosterone target gene (Connell and Davies, 2005; Wulff et al., 2002).

Glucocorticoid and mineralocorticoid can bind to the MR with equal affinity but aldosterone is present in circulation at one hundredth of the concentration of cortisol. To allow aldosterone-specific effects, type 2 11β-hydroxysteroid dehydrogenase is expressed in target tissues to convert cortisol to the inactive form cortisone (or corticosterone to 11-dehydrocorticosterone

in rodents), hence favouring binding of aldosterone (Lombes et al., 1994; Odermatt et al., 2001).

Adrenal androgens, produced in the zR in primates, are known as the C_{19} steroids and consist mainly of dehydroepiandrosterone (DHEA) and its sulphated metabolite DHEA-S. DHEA and DHEAS are precursors of oestrogen and testosterone production. Other adrenal androgens, found in smaller quantities, include androstenedione (A4), androstenediol and 11ß-hydroxyandrostenedione (11OHA) (Rege et al., 2013). Adrenal androgen production begins around the age of 6-8 years at a stage known as 'adrenarche', with an expansion of the zR (Labrie et al., 2005). Adrenal androgens are produced and secreted in large quantities through ACTH stimulation and released into the bloodstream. ACTH binds to MC2R and activates cAMP/PKA dependent pathways to increase STAR, which in turn delivers cholesterol to the mitochondria for DHEA production (Clark and Stocco, 1997; Hammer et al., 2005; Prough et al., 2016). In women, DHEA/DHEA-S is synthesised mostly in the adrenal cortex and some in the ovary. There is also *de novo* DHEA/DHEA-S synthesis in the brain (Maninger et al., 2009). In men, approximately a quarter of circulating DHEA is produced in the testes and adrenal DHEA/DHEA-S provides 50% of all androgens. In premenopausal women, 75% of oestrogens are derived from DHEA/DHEA-S, whereas this is 100% in post-menopausal women (Kroboth et al., 1999; Labrie et al., 2005; Maggio et al., 2015). DHEA-S is readily converted back to DHEA by sulfohydrolases. ACTH is known to be the main control in the production of DHEA but whether alternative factors exist or how this system is regulated remains unclear (Kempná and Flück, 2008).

1.5 The Renin Angiotensin Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is vital in maintaining a normal blood pressure through sodium control (Hall et al., 1990). Renin is released from the juxtaglomerular cells of the kidney into the bloodstream in response to a reduction in renal afferent arteriole pressure (ie renal blood flow) and low plasma sodium concentration. Renin is an aspartyl protease that cleaves angiotensinogen (produced in the liver) to form the decapeptide,

angiotensin I (Ang I). Ang I itself has been shown to stimulate catecholamine release, increase systemic arterial pressure and induce thirst (Miller, 1981). More recently, Angiotensin II (Ang II), generated from the cleavage of Ang I by Angiotensin-converting enzyme (ACE), has been shown to be the main stimulus for thirst and acts to facilitate sympathetic neurotransmission in noradrenaline production via neuropeptide Y, resulting in hypertension (Byku et al., 2010; Westfall et al., 2013).

ACE, an enzyme found on the surface of predominantly lung but also kidney epithelium, then cleaves Ang I to produce the octapeptide angiotensin II (Ang II). Ang II is the physiologically active component and binds to Ang II type I and II receptors (AT1 and AT2). It acts directly on the AT1 receptors in the zG of the adrenal gland to stimulate the production of aldosterone, which promotes active reabsorption of sodium in the distal tubule of the kidney nephron by transcriptionally upregulating SGK1 (serum- and glucocorticoid-induced kinase-1) which causes translocation of the ENaC sodium channel to retain sodium (Chen et al., 1999; Shigaev et al., 2000). SGK1 has also been shown to be recruited by GILZ1 (glucocorticoid-induced leucine zipper protein-1) to form a ENaC-regulatory complex that stimulates ENaC surface expression and activity through further stabilisation of SGK1 by GILZ1 (Soundararajan et al., 2010).

Independent of its effects on aldosterone production, Ang II is also a potent vasoconstrictor of arteriole smooth muscle. Furthermore, Ang II has been shown to act directly on angiotensin II type 1 (AT1) receptors in the proximal tubule to stimulate sodium resorption and alter glomerular filtration rate (Crowley et al., 2006; Lavoie and Sigmund, 2003; Ploth and Gabriel Navar, 1979). Aldosterone too has a direct effect on the vasculature, causing remodelling (Pacurari et al., 2014) as well as nongenomic vasoconstriction through activation of phospholipase C and nitrous oxide release (Arima Shuji et al., 2004).

1.6 Signalling pathways in the developing adrenal cortex

Two key signalling pathways that are known to be involved in adrenal development are the hedgehog (Hh) and Wingless-related integration site (Wnt) pathways. However, signalling pathways involved in zonation and differentiation are less clear. Other factors that are important in adrenal development include WT1 (Wilm's tumour 1), SF-1 (steroidogenic factor 1) and DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1). There is also evidence that ACTH signalling has a role in stem/progenitor cell regulation as well as cortex zonation and maintenance.

1.6.1 The Hedgehog Signalling Pathway

The hedgehog (HH) signalling pathway has 3 hedgehog proteins: Desert hedgehog (DHH), Indian hedgehog (IHH) and Sonic hedgehog (SHH). *Dhh*, *Ihh* and *Shh* are vertebrate homologues of the Drosophila *Hh* gene and are involved in germ cell development, bone development and organ development/stem cell maintenance respectively (Bitgood et al., 1996; Vortkamp et al., 1996).

The *Hh* gene was first discovered in the fruit fly, *Drosophila* melanogaster (Nüsslein-Volhard and Wieschaus, 1980). The HH proteins are produced in the endoplasmic reticulum as precursor proteins that are 45 kDa in length with a 20 kDa amino-terminal (HH-N) and a 25 kDa carboxy-terminal (HH-C). Cholesterol and a palmitoyl moiety is then added to HH-N, to allow HH signalling (Chamoun et al., 2001; Etheridge et al., 2010; Farzan et al., 2008).

For the HH pathway to activate, HH ligands bind to Patched (PTCH), a 12-pass transmembrane receptor, which then relieves PTCH's repression of Smoothened (SMO), another 7-pass transmembrane G protein-coupled receptor (GPCR) and the HH pathway signal transducer across the plasma membrane (Figure 1.3). This allows for SMO to be phosphorylated and activated by Casein kinase 1α ($CK_{1\alpha}$) and GPCR kinase 2 (GRK_2) (Chen et al., 2002, 2011; Pietrobono et al., 2019; Wilson et al., 2009). In the absence of Hh, Smo is repressed and Gli2 and Gli3, GLI (glioma-associated) transcriptional factors, which are a family of zinc finger

transcription factors, are proteolytically cleaved to form repressors which inhibit expression of Hh regulated genes. There are 3 *GLI* genes in mammals; *GLI1*, *GLI2* and *GLI3*. In the presence of activated SMO, are no longer cleaved to form the repressive forms and full length GLI2 can act as a transcriptional activators and induce expression of Gli1 which is the primary transcription factor for activation of transcription of HH target genes (Chen et al., 2011; Pietrobono et al., 2019). As Gli1 is only expressed in cells receiving a Hh signal, its presence is used as a marker of HH signalling pathway activation (Gupta et al., 2010; Vokes et al., 2007).

HH pathway can be manipulated with agonists and antagonists by changing the conformation of SMO. HH pathway agonists include purmorphamine and smoothened agonist (SAG) and its main antagonist is cyclopamine (Wang et al., 2009).

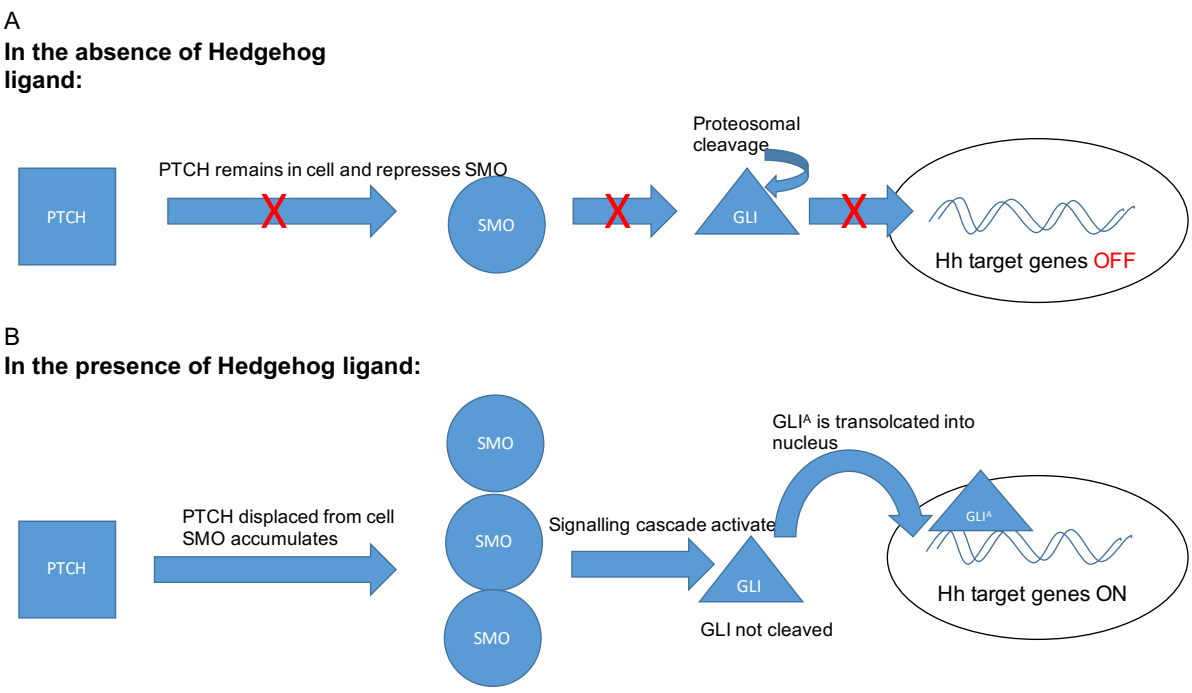

Figure 1.3 Hedgehog (Hh) signalling pathway
(A) In the absence of HH ligand, PTCH (Patched) remains in the cell membrane and represses SMO (Smoothened) activity. As a result, GLI undergoes proteasomal cleavage, resulting in the repressor form (GLI^R) being translocated into the nucleus. No HH target gene translation occurs. (B) Upon HH ligand binding, PTCH is displaced from the cell membrane and SMO is activated and accumulates in the primary cilium. SMO activation leads to a signalling cascade to allow for translocation of activated GLI (GLI^A) into the nucleus and HH target genes are expressed.

The HH signalling pathway has been identified to play a major role in adrenal development and the maintenance of a subcapsular stem cell population of the adrenal gland, that are *SHH* positive (King et al., 2009). *Shh* null mice are non-viable, dying of severe developmental defects (Chiang et al., 1996; Stone et al., 1996). *Shh* is detected from E11.5 in mice, possibly initiated by *Sf-1* expression and sits in the subcapsular region of the adrenal cortex (King et al., 2009). *Shh* co-localises with *Sf-1* in the subcapsular region only in non-steroidogenic cortical cells. Conditional *Shh* null mice in which *Shh* is knocked out from steroidogenic tissues, including the adrenal, by cre recombinase under the control of the *Sf-1* promoter, have adrenal hypoplasia where normal function and zonation is maintained but with reduced proliferation and a significantly thinner adrenal capsule (Ching and Vilain, 2009; King et al., 2008). This

indicates that the role of *Shh* is in cell proliferation and maintenance of the adrenal cortex stem/progenitor cells rather than initiation of cell differentiation and population of the separate cortical zones (Huang et al., 2009; Laufer et al., 2012).

Shh is expressed in the subcapsular region, whereas its receptor, PTCH and activated transcription factor GLI1 are located in the capsule (King et al., 2009). Studies using *Gli1*-Cre recombination and *Shh* LacZ reporter allele performed by King *et al.,* identified a dual lineage model of adrenal development (a primary and secondary lineage). They showed that the cells of the primary lineage are SF1 positive and derived from the coelomic epithelium The secondary lineage cells are SF1 negative and of mesenchymal origin. The primary lineage cells then initiate *Shh* expression to convert neighbouring secondary lineage mesenchymal cells to become steroidogenic cells and this is achieved through Shh signalling (King et al., 2009). This correlates with the fact that *Shh* KO adrenals are smaller but functional (Ching and Vilain, 2009).

1.6.2 WT1, DAX1 and ACTH signalling

The Wilm's tumour suppressor gene 1 (*Wt1*) is one of the earliest genes expressed within the AGP and with another transcriptional co-factor Cited2 (CREB-binding protein/p300-interacting transactivator, with ED-rich tail, 2) has been shown to activate *Sf-1* expression. It has been shown that in *Wt1* KO mice, there is no *Cited2* or *Sf-1* expression (Val et al., 2007; Wilhelm and Englert, 2002). SF-1 is an orphan nuclear receptor and is a transcriptional regulator of genes encoding steroidogenic enzymes (Luo et al., 1995). After activation of SF-1 and separation of the AGP, the adrenocortical cells lose *Wt1* expression, while the gonadal primordium retains both *Sf-1* and *Wt1* expression. Bandiera *et al*., showed that overexpression of *Wt1* blocks the differentiation process into steroidogenic cells. They generated mice that allowed Cre-mediated activation of WT1. Specifically, they studied 2 isoforms of WT1 generated through alternative splicing of exons 9 and 10 to generate an isoform that includes or excludes three critical amino acids (lysine, threonine and serine) between the zinc fingers 3 and 4. These isoforms were named WT1+KTS or WT1-KTS. The mice generated in this way had WT1 activation in a tissue specific manner (Bandiera et al., 2013; Barbaux et al., 1997;

Bor et al., 2006; Hastie, 2017). Both isoforms are important in development, as global KO mice of either isoform die in utero (Hammes et al., 2001). The WT1-KTS isoform is a transcriptional regulator and has a high affinity for DNA (Bickmore et al., 1992) whereas the WT1+KTS preferentially bind to RNA (Caricasole et al., 1996). The WT1+KTS mice had normal adrenal architecture and the WT1-KTS mice had small adrenal glands with abnormal morphology. Further analysis of these glands showed a reduction in the size the gland but the zonation and function of the cortex was maintained except for a dramatic reduction of the X-zone. Lineage experiments with WT1 and GLI1 E18.5 embryos demonstrated only partial overlap of expression of these two genes and that WT1 and GLI1 expression are likely to denote a different subpopulation of cells within the adrenal capsule. Further experiments on adult *Wt1*:Cre-ERT2 mice, activated with tamoxifen, showed that at 7 months after tamoxifen injection, a group of GFP labelled cells retained WT1 expression and remained within the adrenal capsule whilst many of the other cells that have migrated into the cortex have lost WT1 identity and gained steroidogenic features with SF1, AKR1b7 and 3βHSD2 expression (Bandiera et al., 2013).

DAX1, an orphan nuclear hormone receptor, co-localises with SF-1 at the urogenital ridge at E9.5 (Ikeda et al., 1996). However, its role in differentiation is unclear and young *Dax1* KO mouse have enhanced steroidogenesis (Babu et al., 2002). With aging the adrenal glands of these mice becomes dysplastic and they develop adrenal failure, with premature depletion of the pool of progenitor cells (Scheys et al., 2011). The phenotype of older *Dax1* KO mice is in line with humans with *DAX1* mutations who develop X-linked adrenal hypoplasia congenita (AHC), resulting in primary adrenal failure (Muscatelli et al., 1994; Scheys et al., 2011).

ACTH signalling is another pathway implicated in adrenal stem cell regulation (El Ghorayeb et al., 2016). The importance of its role in adrenal development was shown in MC2R (Chida et al., 2007) and *Mrap* knockout mice, which both show a significantly reduced adrenal gland with a thickened adrenal capsule. The absence of ACTH signalling in these mice led to an expansion of the capsular stem cell niche. The subcapsular cells and throughout the cortex were *Shh*, *Wnt4* positive. A proportion co-expressed with CYP11B2 staining pointing to zG status (Novoselova et al., 2018). ACTH signalling in the adrenal gland has been shown to

repress the WNT/β-catenin pathway, via cAMP/PKA activation, for zF conversion by lineage conversion (Drelon et al., 2016a). The *Mrap* KO mice has an intact functional zG but an absent zF and a concentric zone between the zG and medulla that are negative for *Cyp11b1*, *Cyp11b2* and *20-αHSD*. These cells were WNT4/β-catenin positive but not for the downstream targets of WNT signalling, namely Lymphoid enhancer factor (LEF1) and Disabled homolog 2 (DAB2), suggesting that these cells may have never had or lost their zG identity (Novoselova et al., 2019, 2018).

1.6.3 The Wnt Signalling Pathway

WNT signalling is known to be involved in development, cell fate specification and differentiation and stem cell maintenance (Logan and Nusse, 2004). WNT signalling takes place via canonical and non-canonical pathways. The canonical pathway regulates β-catenin, which is a transcriptional co-activator. In the absence of WNT signalling, the amino terminal region of β-catenin is bound by the destruction complex which includes the tumour suppressors Axin and adenomatous polyposis coli (APC) and kinases casein kinase 1β (CK1β) and glycogen synthase kinase-3β (GSK-3β). β-catenin is sequentially phosphorylated by CK1β and GSK-3β, which creates a substrate for the E3 ubiquitin ligase β-TrCP, also part of the complex. The ubiquitinated molecule is then degraded by proteasomes. This process is essential in the maintenance of a low level of β-catenin to prevent activation of WNT target gene transcription, which is under the repressive influence of the T cell-factor/lymphoid enhancer factor (TCF/LEF) and Groucho/TLE co-repressor complex. During WNT signalling, the ligand binds to Frizzled, (Fz), a GPCR. Fz also interacts with the transmembrane proteins LRP5 or LRP6 (low density lipoprotein receptor related protein) which acts to further stabilise the complex. This complex then recruits dishevelled (DVL), which inhibits the phosphorylation of β-catenin by GSK-3β, allowing β-catenin to dissociate from the destruction complex. This process will lead to an accumulation of free β-catenin in the cytoplasm which then translocates into the nucleus. Once within the nucleus, β-catenin, a co-activator, binds with TCF/LEF displacing the co-repressor complex, thus enhancing the expression of WNT target genes (El Wakil and Lalli, 2011; Huang and He, 2008; Zeng et al., 2008).

The non-canonical pathway of WNT does not involve β-catenin and is known as the WNT/JUN N-terminal kinase (JNK) or WNT/calcium pathway. The endogenous antagonists to this pathway are Frizzled related proteins (sFrps) which binds to WNT proteins (Kawano and Kypta, 2003) and DKK, part of the Dickkopf family of secreted proteins, which inhibit WNT signalling by binding to the LRP5/6 co-receptors (Niehrs, 2006; Rao and Kuhl, 2010).

In the mouse adrenal cortex, WNT4 and β-catenin are seen in the adrenocortical primordium (AP) at E11.5 (Heikkila et al., 2002) and E12.5 (Kim et al., 2008) respectively, whereas *Sf-1* is expressed in the urogenital ridge at E9.5 (Parker and Schimmer, 1997) and remains expressed in the AP after its separation from the gonads (Ikeda et al., 1994). As the mouse develops, *β-catenin* expression, which initially overlaps with *Sf-1* expression throughout the adrenal cortex, becomes exclusively subcapsular by E18.5 (Kim et al., 2008). Complete *β-catenin* KO is embryonically lethal therefore conditional KO models were used to study the adrenal gland instead (Haegel et al., 1995; Kim et al., 2008). In a partial adrenal *β-catenin* KO mouse model, where *β-catenin* is deleted in Sf-1 expressing cells, the adrenal glands were aplastic by E18.5 with depletion of adrenocortical cells as the mice aged. β-catenin and Sf-1 have been shown to act in synergy within the cortex to activate target genes (Gummow et al., 2003; Mizusaki et al., 2003), suggesting that β-catenin may have a role in steroidogenesis.

WNT 4 appears to be a key factor in cortical differentiation and zonation, especially in the zG, where it is located and functionally active. *Wnt4* mutant mice have reduced *Cyp11b2* and *Dlk1* expression and profoundly lowered aldosterone production. *Wnt* 4 is present in the mouse adrenal from E11.5 but its role in development is less well defined (Heikkila et al., 2002). WNT 4 also plays a significant role in humans, as a loss of WNT 4 function leads to SERKEL syndrome, with organ dysgenesis including the adrenals (Mandel et al., 2008). Recently, RSPO3 (R-spondin-3, which activates the canonical WNT signalling pathway) appears to play a key role in initiating adrenal progenitor cell differentiation and possibly cortical zonation. RSPO3 binds to LGR receptors in the adrenal capsule and activates β-catenin signalling to determine and maintain glomerulosa cell fate throughout life. Upon activation of the pathway via WNT4, *Shh* is activated to initiate steroidogenesis via *Gli1*. Without RSPO3, SHH signalling is not present, resulting in impaired adrenal growth and inability to maintain the zG

as well as a reduction in Cyp11b2 expression and ultimately decreased aldosterone production (Vidal et al., 2016). The Wnt/β-catenin is also dependent on ZNRF3 (zinc and ring finger 3) for adrenal homeostasis. Basham *et al.,* demonstrated that *Znrf3* is expressed beneath the *Rspo3* expressing cells, throughout the adrenal cortex and that the loss of ZNRF3 results in adrenal hyperplasia, favouring the zF phenotype (Basham et al., 2019).

Studies on the WNT pathway show that WNT signalling, unlike SHH, may have a contribution to adrenal cortex zonation. In the adrenal *ß-catenin* KO mouse model, where only 50% of *ß-catenin* was knocked out in adrenocortical cells, adrenal development continued as normal at birth but the adrenal began to fail after birth with progressive cortical thinning, reduced steroidogenic capacity and a loss of progenitor cells (Kim et al., 2008, 2009). On the other hand, exaggerated increase in WNT pathway signalling has recently been shown to cause over expansion of the zG and hyperaldosteronism, indicating a direct effect of WNT signalling on the zG (Berthon et al., 2010).

WNT/β catenin is also able to inhibit zF differentiation suggesting that it promotes progenitor cells to differentiate into zG cells rather than zF cells (Walczak et al., 2014). It has been shown that PKA stimulation can decrease ß-catenin activation and can be a driver of WNT inhibition in the zF to allow zF differentiation (Drelon et al., 2016a).

PKA activation has been shown to be important in cell conversion from zF to zR, via lineage conversion of the innermost zF cells. This was achieved by the creation of a constitutive PKA activation model with genetic deletion of Protein Kinase cAMP-Dependent Type I Regulatory Subunit Alpha (*Prkar1a*). This lineage conversion appears to be sexually dimorphic: testicular androgens antagonise PKA to increase WNT signalling and delay adrenocortical cell turnover and zR formation, whereas mice with gonadectomies resulted in zG like formation and hypercortisolism (Dumontet et al., 2018). In females, adrenocortical cell turnover was 3-fold higher than males and females have additional stem/progenitor compartments in the adrenal capsule (Grabek et al., 2019).

Another inhibitory effect of WNT signalling on the zF can be seen with *Mc2r* expression, which has been suggested to be important in zF zonal patterning (Drelon et al., 2016a). There is *Mc2r* and *Mrap* expression throughout the rat adrenal cortex, with weak expression in the zG and higher expression in the zF. Its principal site of action is in the zF to generate glucocorticoids in response to ACTH (Gorrigan et al., 2011; Novoselova et al., 2019). However, in the acute setting, ACTH is known to stimulate aldosterone production and can also stimulate DHEA production in adrenal cells (Clark et al., 2016; El Ghorayeb et al., 2016; Funder, 2016). Interestingly, as the zG has low levels of StAR and Cyp11b1, aldosterone production via ACTH is speculated to be from cells within the zF that become zG cells and migrate to the zG to function (Vinson, 2003). In the rat adrenal gland *Mrap* and *Mc2r* are expressed in the UZ (Gorrigan et al., 2011), pointing to a role in progenitor cell regulation. *Mc2r* KO mice have an atrophied zF but a histologically normal zG, indicating that in the absence of ACTH signalling the zG alone cannot sustain a zF phenotype or produce corticosterone (Chida et al., 2007). In humans, *MC2R* and *MRAP* mutations result in familial glucocorticoid deficiency (FGD). FGD patients have an atrophied zF and require lifelong glucocorticoid replacement but is not mineralocorticoid deficient (Clark and Weber, 1998).

More recently, the histone methyltansferase Enhancer of Zeste Homolog 2 (EZH2) have been shown to have a role in adrenal gland zonation and development (Drelon et al., 2016b). Targeted adrenal inactivation of *Ezh2* in mice through the use of *Sf1Cre* showed adrenal hypoplasia with primary glucocorticoid deficiency and high ACTH levels and reduced zF differentiation. Aldosterone production appeared normal in females but low in males (Mathieu et al., 2018).

Transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) is another novel factor implicated in adrenal gland development and zonation. Adrenal specific loss of Znrf3, driven by *Sf1*Cre, resulted in adrenal hyperplasia with the expansion of the zF, induced a moderate activation of Wnt/ß-catenin signalling, maintaining the inner cortex histologically and functionally (Basham et al., 2019).

1.7 Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) were first identified in 1965 by Urist whilst investigating rat bone and cartilage formation (Urist, 1965). BMPs are members of the Transforming Growth Factor beta (TGF-β) family of cytokines that have been shown to be involved in a multitude of processes including the regulation of growth, differentiation and chemotaxis (Chang et al., 2002; Nohe et al., 2004; Xiao et al., 2007). They are also involved in the mediation of programmed cell death and apoptosis (Chen et al., 2004), as well as the regulation and maintenance of stem cells (Munoz-Sanjuan and Brivanlou, 2002; Ying et al., 2003).

Many *Bmp* knockout rodent models show disruption in development, such as heart defects and limb shortening. BMPs have been linked with many types of cancers (Bach et al., 2017; Thawani et al., 2010; Zhang et al., 2016). Within the adrenal gland, BMPs, especially BMP4, have been shown to be expressed in adrenocortical cells (Farnworth et al., 2006a, 2006b; Vinson, 2016) and to affect steroidogenesis, by decreasing DHEA, DHEAS and androstenedione production (Rege et al., 2015).

There is evidence of BMP crosstalk with other signalling pathways. For example, BMP regulation can have an effect on Shh induced proliferation (Bhardwaj et al., 2001). Shh and BMPs are also known to co-expressed at many sites (Bitgood and McMahon, 1995). BMPs interact with the Wnt pathway and may be involved in many other biological processes (Hiyama et al., 2011; Perez et al., 2011). BMPs can act as morphogens as demonstrated in drosophila wing patterning (Nellen et al., 1996). In humans, there is evidence of a morphogenic effect in the growth plates of bones (Nilsson et al., 2007).

BMP is the mammalian homologue of the drosophila morphogen decapentaplegic (Dpp). There are more than 20 BMPs discovered so far and they are further subdivided into groups according to structure and amino acid similarity. BMPs are distinct within the TGF-ß superfamily as they have seven cysteine residues, of which 6 build a cysteine knot and the seventh is used for dimerization (Allendorph et al., 2011; Hinck and Huang, 2013; McDonald

and Hendrickson, 1993). BMPs are synthesised as large precursor molecules and are secreted proteins. BMPs have a signal peptide, pro-domain and a mature peptide. The signal peptide is cleaved first at a dibasic site, releasing the C-terminal active domain. This is then glycosylated and dimerised. The pro-domain is then cleaved and the mature peptide is secreted following proteolytic cleavage to yield a carboxy-terminal mature protein dimer that is bioactive. The mature BMP is secreted as a homodimer or heterodimer (formed with other members of the BMP family) (Hogan, 1996; Xiao et al., 2007).

All BMP ligands bind to serine-threonine kinase receptors forming a hetero-oligomeric complex of type I and II receptors (Figure 1.4) (Kawabata et al., 1998). In mammals, there are seven type I and five type II receptors. Of the seven Type I receptors (ALK1-7) for the TGF-β family of ligands, only three bind to BMP: type 1A BMP receptor (BMPR1A or ALK3), type 1B BMP receptor (BMPR1B or ALK6) and type 1A activin receptor (ActR-1A or ALK2). Three out of four Type II receptors for the TGF-β family interact with BMPs: type 2 BMP receptor (BMPR2), type 2 activin receptor (ActR2A) and type 2B activin receptor (ActR2B) (Wang et al., 2014). Both type I and type II receptors have a short extracellular domain with several conserved cysteine residues and a single transmembrane domain. The intracellular domain contains a serine-threonine region (Yamashita et al., 1996). When BMPR2 receptors bind with a BMP ligand, activation of the BMPR1 receptor occurs via phosphorylation, forming a heterotetrameric complex, allowing signal transduction to the nucleus (Blanco Calvo et al., 2009). Although BMP receptors are limited in type, the diversity of BMP function is immense. This diversity is due to different affinities of the BMPs to the receptors and that different cell types express the receptors and downstream components in different quantities and combinations (Kawabata et al., 1998).

The Smad family of transcription factors are responsible for signal transduction and the family is divided into three distinct groups. Upon activation, a type I receptor will then phosphorylate the R-Smads (receptor-regulated Smads) of which Smad 1, 5 and 8 act in the BMP signalling pathway. The R-Smad undergoes phosphorylation and is released into the cytoplasm. The phosphorylated R-Smad is then recruited by the only Co-Smad (common Smad), Smad 4, to form the Smad complex. This complex is then translocated into the nucleus and upon binding

with co-activators or co-repressors modulates expression of downstream target genes (Wang et al., 2014).

Smad 6 and Smad 7 are I-Smads (inhibitory Smads) (Nakao et al., 1997; Zhang et al., 1996) and act by stably binding to type I receptors and preventing phosphorylation of the R-Smads, hence halting the pathway (Imamura et al., 1997). I-Smad is also able to interact with Smurf-1 (Smad ubiquitin regulatory factor 1) and smurf-2 to modulate Smad proteins for proteosomal and lysosomal degradation (Kavsak et al., 2000; Murakami et al., 2003; Xiao et al., 2007).

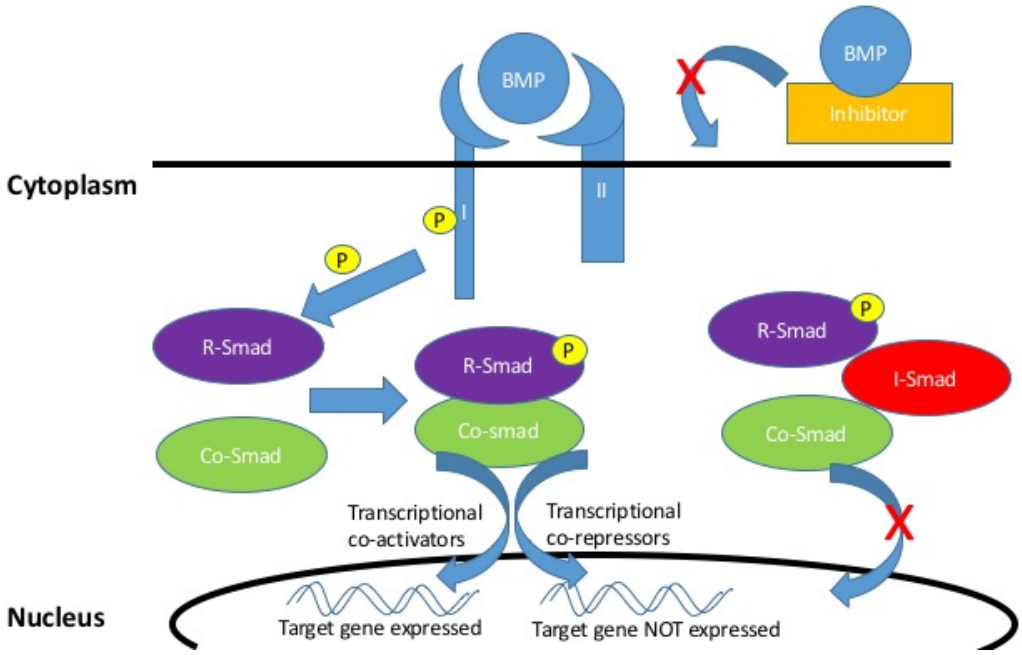

Figure 1.4 Bone morphogenetic protein signalling pathway
Upon binding of Bone morphogenetic protein (BMP) ligands with its receptors, BMPRI (Bone morphogenetic protein Receptor I) and BMPRII (Bone morphogenetic protein Receptor II), BMPRII phosphorylates and activates BMPRI. Phosphorylated BMPRI subsequently phosphorylates receptor-activated Smad proteins (R-Smads). The R-Smad will then associate with common mediator-Smad (Co-Smad) and enter the nucleus to regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression. Endogenous extracellular antagonists (inhibitors), such as noggin, bind BMP ligands so it cannot bind with BMP receptors. Inhibitory Smads (I-Smad) works intracellularly to prevent R-smad and co-smad association and hence cannot enter the nucleus for target gene expression or repression.

Extracellularly, BMPs are regulated by a host of endogenous antagonists, such as follistatin, which was originally thought to be activin–binding but increasing evidence reveals that it exerts its antagonistic effect by directly binding non-competitively to the BMP-receptor complex, forming a trimeric complex (Iemura et al., 1998; Weisinger et al., 2008). A large group of BMP antagonists has been classified according to the size of their cysteine knots: the DAN family (eight membered ring) which includes Dan, Cerberus, Coco, Protein related to DAN and Cerberus (PRDC), Gremlin, USAG-1 and sclerostin; Twisted gastrulation (tsg) has a nine-membered ring; and chordin and noggin form the third group with ten-membered rings. They act by direct binding to BMP ligands to form inactive complexes (Avsian-Kretchmer and Hsueh, 2004; Yanagita, 2005). However, there has been a debate about whether USAG-1 and sclerostin form a separate group as they are secreted as monomers while many of the others form dimers and they do not contain extra cysteine residues to form homodimers (Avsian-Kretchmer and Hsueh, 2004; Groppe et al., 2002; Kusu et al., 2003).

Below, the individual BMPs discussed are the ones that have been shown to be present in the adrenal gland (Inagaki et al., 2006; Johnsen and Beuschlein, 2010; Johnsen et al., 2009; Mitsui et al., 2014; Otani et al., 2010; Rege et al., 2015). BMP3 is not expressed in the adrenal gland and has not been shown in published data to play a role in adrenal gland physiology.

1.7.1 BMP3b

BMP3b and GDF 10 are synonymous terms used for this protein. *BMP3b* is located on chromosome 10 and encodes a 478 amino acid protein. It was first identified as a new member of the transforming growth factor-beta (TGF-β) in 1995, showing a high similarity to BMP3, with 83% amino acid identity in the mature C-terminal region and only 30-35% homology in the pro-region. Together, BMP3 and BMP3b form their own subgroup within the TGFβ superfamily (Cunningham et al., 1995). *Bmp3b* was first cloned from rats in 1996 by the Kangawa group in Japan (Takao et al., 1996). Other studies have shown the importance of Bmp3b in brain (Cunningham et al., 1995), skeletal and uterine development (Zhao et al., 1999). The same Japanese group was able to clone the human *BMP3b* gene later on in the same year. The human *BMP3b* gene is found at chromosome 10q11.22 and spans about 13 kilobases and has 3 exons and 2 introns. The precursor protein has 478 amino acids, with a

molecular weight of 53,122 Daltons. By proteolytic cleavage of the precursor protein at the putative polybasic proteolytic processing site containing Arg-Arg-Lys, a mature, biologically active, BMP3b protein is generated. This mature protein is 110 amino acids long with one N-glycosylation site and contains the carboxy-terminal region which has 7 conserved cysteine residues. *BMP3b* is highly conserved through species as the mature protein is 98% similar to rat *Bmp3b*. From this original paper, *Bmp3b* was shown to be expressed in many organs other than bone, especially brain, lung, skeletal muscle, pancreas and testes, with little expression in the heart and prostate (Hino et al., 1996).

In 1999, Zhao *et al*., produced a *Bmp3b* KO mouse model to determine its biological function. This mouse was generated by using a targeting construct that replaced endogenous *Bmp3b* exon 2 and 3 with a neo cassette resulting in deletion of the BMP3b C-terminus. The resulting *Bmp3b* KO mice were all viable with normal reproduction capacity, comparable to wild type littermates. Despite high expression of *BMP3b* in mouse cerebellum, skeleton and uterus, the KO mouse phenotype appeared normal during development of all these organs. As *Bmp3* and *Bmp3b* share 83% similarity, compensatory upregulation and function of *Bmp3* was postulated to explain the normal development (Zhao et al., 1999). However, only a few tissues were investigated and the adrenal glands were not studied in this animal.

1.7.2 BMP2

BMP2 is found on chromosome 20 and encodes a 396 amino acid protein. BMP2 is a potent inducer of osteoblastic activity, with the ability to induce all markers of osteoblast activity (Cheng et al., 2003) and has been used in its recombinant form for fracture healing (Govender et al., 2002; Tsuji et al., 2006). Bmp2 is essential for development as *Bmp2* KO mice is embryonically lethal and its expression is found in many other organs such as the heart and brain (Zhang and Bradley, 1996). Within the brain, Bmp2 induces medulloblastoma cell apoptosis, which can be blocked by noggin (Hallahan et al., 2003). Within the adrenal gland, it has been reported to have reduced expression in adrenocortical carcinomas (Johnsen et al., 2009) and overexpression of *BMP2* has been seen in rare cases of adrenal myelolipomas (Mitsui et al., 2014).

1.7.3 BMP4

BMP4 is a highly conserved protein in evolution. *BMP4* is located on chromosome 14 and encodes a 408 amino acid protein. It is involved in many developmental processes, including the development of the dorsal-ventral axis (in synergy with *Shh* expression) (Liem et al., 2000), bone, teeth (Jia et al., 2013), eyes (Bakrania et al., 2008), heart (Jiao et al., 2003) and blood (Sadlon et al., 2004).

Within the adrenal gland, *Bmp4* is expressed throughout all the zones, with highest expression within the zG (Huber et al., 2008; Rege et al., 2015). However, its function appears to be mainly related to androgen production for the zR and regulation of the medulla. Rege *et al.,* overexpressed *BMP4* in H295R cells and found that it suppressed C19 steroid synthesis via P450c17, resulting in reduction of DHEA, DHEA-S and androstenedione (Rege et al., 2015). In the chick embryo, Huber *et al.,* found that *Bmp4* was essential in the early induction of neuronal and catecholaminergic phenotypes in neural crest cells. BMP4 appears to augment the number of tyrosine hydroxylase (TH) positive cells by inducing TH in TH negative cells and not by proliferation, hence it influences cells to become chromaffin cells (Huber et al., 2008).

1.7.4 BMP5

BMP5 is within the subfamily of BMPs which include other members BMP2, 6 and 7. The *BMP5* gene is located on chromosome 6 in humans and encodes a 454 amino acid protein. Its role includes generation of osteoclasts for bone regeneration (Wutzl et al., 2006) and dendritic growth in neurons (Beck et al., 2001). In the adrenals, it has been shown to have reduced expression in adrenocortical carcinoma cell lines (Johnsen et al., 2009). There is also a reduced expression of BMP5 in other cancers including colorectal, pancreatic and non small cell lung cancer (Chen et al., 2018; Deng et al., 2015; Virtanen et al., 2011). However, there is increased *BMP5* expression in lung adenocarcinoma (Deng et al., 2015).

1.7.5 BMP6

The *BMP6* gene is found on chromosome 6 in humans and it encodes a 513 amino acid protein. It is similar to other members of its subfamily in that it will induce all markers of osteoblastic

differentiation, as shown in mesenchymal progenitor and osteoblastic cell models (C3H10T1/2, C2C12 and TE-85 cell lines) (Cheng et al., 2003). *Bmp6* null mice are viable and fertile. The only defect noted is of delayed ossification of the sternum (Kugimiya et al., 2005; Perry et al., 2008; Solloway et al., 1998). Further studies have shown that *Bmp6* null mice had haemochromatosis where iron is deposited on organs such as the liver (Meynard et al., 2009). The KO mice are hyperglycaemic with reduced insulin production due to a decrease in pancreatic islet cell number, hence indicating *that Bmp6* may have a role in glucose regulation in diabetes mellitus. In support of this, treatment of NOD (Non Obese Diabetic) mice or ob/ob (obese/obese) with recombinant Bmp6 (rhBmp6) for 7 days resulted in a reduction in glucose levels and improved glucose clearance, lipid profile and increased bone volume (Paralkar et al., 2010).

Within the adrenal cortex, *BMP6* is linked to aldosterone synthesis. BMP6 treatment of H295R cells augments Ang II induced *CYP11B2* transcription and ultimately increases aldosterone production by activation of SMAD 1,5,8 through binding to ALK2/3 with ActRII resulting in an enhancement of Ang II induced MAPK activation. However, BMP6 has no effect on potassium-induced aldosterone production (Inagaki et al., 2006). BMP6 is also found to act through receptors of the BMP pathway, namely ALK2, ALK3 and type 2 activin receptors. BMP6 is thought to enhance the induction of ERK1/2 phosphorylation by AngII which would increase activation of CYP11B2 leading to increased aldosterone production (Inagaki et al., 2006).

1.7.6 BMP7

BMP7 is a 431 amino acid polypeptide, with its gene found on chromosome 20. Like BMP5, it is able to elicit dendritic growth in rat neurons (Withers et al., 2000) and is also able to induce all markers of osteoblastic differentiation (Cheng et al., 2003). Zeisberg *et al.,* found that the presence of Bmp7 can reduce cardiac fibrosis (Zeisberg et al., 2007) and Tseng *et al.,* showed evidence that Bmp7 can promote differentiation of brown adipocytes and thermogenesis, with a potential role in obesity treatment (Tseng et al., 2008). *Bmp7* null mice have severe renal and ocular defects (Dudley et al., 1995) as well as impaired skeletal axis and retarded bone ossification (Jena et al., 1997). The adrenals and gonads were observed to be normal though

the mice were not viable beyond a maximum of 10 days, with the majority dying at day 1 (Dudley et al., 1995). In cases of phaeochromocytoma (tumours of the adrenal medulla), *BMP7* is overexpressed, resulting in increased cell division and migration (Leinhauser et al., 2015).

1.8 BMP Signalling in the adrenal

There is some evidence of the role of BMP signalling in adrenal steroidogenesis and possibly development (Johnsen and Beuschlein, 2010). However, direct evidence is difficult to gather as many animal models that modify the BMP signalling pathway die in utero (Wang et al., 2014). *In vitro* evidence suggests TGFβ1, BMP6, activins and inhibins play a role in steroidogenesis in the human adrenocortical cell line, H295R cells (Hotta and Baird, 1986; Liakos et al., 2003; Suzuki et al., 2004; Vanttinen et al., 2003). TGFβ1 inhibits both aldosterone and cortisol production via CYP11B2 and CYP11B1 respectively in H295R cells as described by Liakos *et al.,* (Liakos et al., 2003). Suzuki *et al*., demonstrated that BMP6 can stimulate the transcription of *CYP11B2* by activating the Smad1/5 pathway through Type I and Type II receptors in H295R cells (Suzuki et al., 2004). Activin A treatment of H295R cells inhibited *STAR* which led to a reduction in *CYP17* mRNA expression, resulting in a reduction in androstenedione, DHEA, DHEAS and cortisol production (Vanttinen et al., 2003). Interestingly, Booroola sheep have a naturally occurring mutation of *Bmpr1b* gene leading to a single amino acid substitution of arginine at amino acid postion 249 to a glutamine residue (Q249R). The adrenal glands from these sheep are small, whilst all other organs such as heart, liver, lungs and kidneys are normal, suggesting a role for *Bmpr1b* in adrenal development (Fabre et al., 2003; Souza and Baird, 2004; Souza et al., 2001).

1.8.1 Microarray of the rat adrenal cortex

The involvement of BMPs in adrenal gland development and physiology is further supported by unpublished microarray data from the King group. The rat adrenal gland has a natural fracture plane between the zG and zF. Two adult male Sprague Dawley rat adrenals were

squashed between 2 glass slides which separated the adrenal gland into 2 parts: capsule/zG cells and zF/medulla cells (King et al., 2009). The 4 samples (2 of each section) were snap frozen in liquid nitrogen and RNA was extracted and first strand cDNA synthesis was performed with primers designed to introduce an RNA polymerase sequence. This was then used to direct RNA synthesis and the cRNA populations were analysed on the rat genome Affymetrix RG34A chips. Data was analysed by comparing the differences in expression in the outer and inner zones, with *AT1* and tyrosine kinase expression demonstrating the successful separation of these zones.

Among the microarray results, *Bmp3b, Gdf11, Smad-7* and follistatin-like, *Bmpr1a* and activin receptors along with *Shh* and *Dax1* were all overexpressed in the capsule/zG whereas *Bmp2, Bmp3, Gdf-8* and *Gdf-15* were more highly expressed in the inner zone. To verify these findings, real-time qPCR was performed with each of the BMP components (work performed by ex-PhD student Dr. Bakmanidis, Figure 1.5). The most significant difference was that *Bmp3b* was exclusively expressed in the outer zone and *Bmp2* had the biggest difference in expression between the 2 zones, being highest in the inner zone.

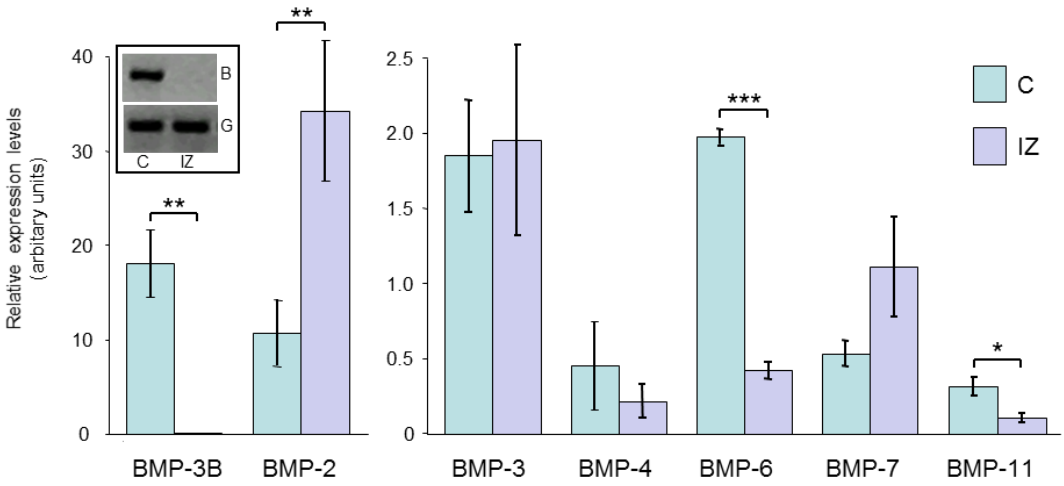

Figure 1.5 Real time qPCR of BMPs in rat adrenals
Real time qPCR analysis of BMPs in capsular (capsule and zG) and inner zone (zF and medulla) cDNA from Sprague Dawley rat adrenals normalised against GAPDH. Inset shows end point PCR analysis of BMP3b (B) run on an agarose gel, with GAPDH (G) as a house keeping gene. C=capsular zone, IZ = Inner zone. * $p<0.05$, ** $P<0.01$, *** $P<0.001$

1.8.2 Expression of BMPs and BMP pathway components in H295R cells

In H295R cells, a human adrenocortical carcinoma cell line, the role of BMP6 in aldosterone production has previously been published (Inagaki et al., 2006, 2007; Otani et al., 2010; Suzuki et al., 2004). Suzuki *et al.,* showed that BMP6 seemed to augment Angiotensin II-induced aldosterone production and act through receptors of the BMP pathway, namely ALK2, ALK3 and activin type II receptors. However, BMP6 was not shown to be relevant in potassium induced aldosterone production (Suzuki et al., 2004).

The role of BMPs in adrenal development, zonation, steroidogenesis and tumorigenesis is still not clearly defined. Following on from the rat expression data, shown in Figure 1.5, Dr Bakmanidis showed that both *BMP3b* and *BMP2* mRNAs were present in H295R cells. The receptors for BMP3b and BMP2 were also present. The finding of BMP receptor expression in H295R cells is consistent with published data (Inagaki et al., 2006).

Using a luciferase reporter assay containing a promoter driven by a BMP responsive element, upstream of luciferase, Dr Bakmanidis showed that BMP3b was able to inhibit the transcriptional activity of BMP2 and that BMP3b has no effect on this promoter in the absence of BMP2 (Figure 1.6), indicating that there could be a direct suppressive effect of BMP3b on BMP2 activity. Furthermore, Figure 1.7 shows a significant reduction in the mRNA expression of *BMP2* in H295R cells that have been stably transfected with *BMP3b*. Together these data demonstrated that BMP3b can inhibit both BMP2 expression and activity.

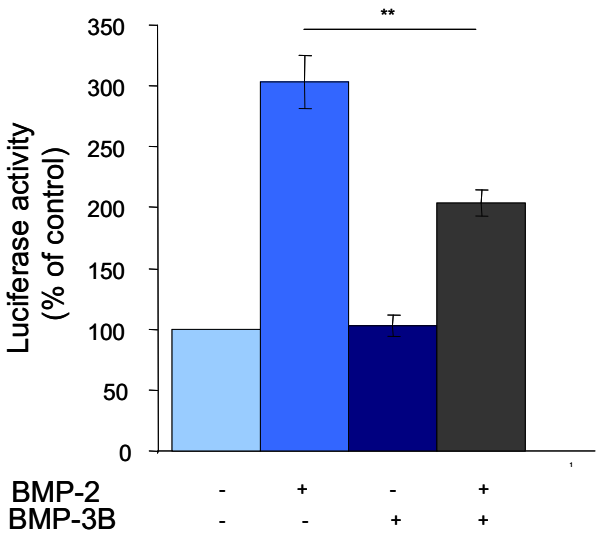

Figure 1.6 Transfection of H295R cells with Xvent2-luc
Xvent2-luc contains a promoter driven by a BMP responsive element, that is activated by BMP2, leading to luciferase expression. ** P<0.01
Graph taken from work by Dr A Bakmanidis.

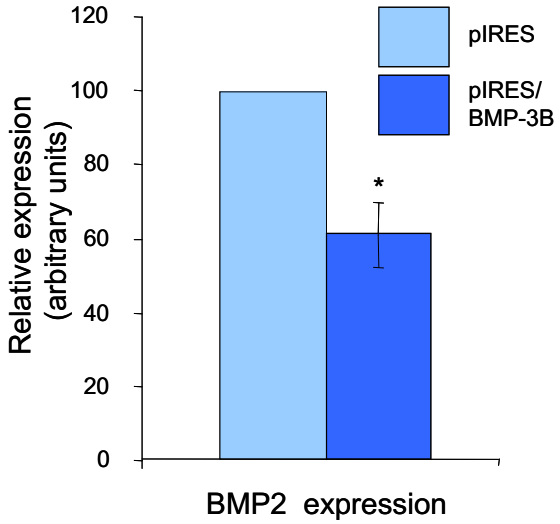

Figure 1.7 Real time RT PCR of BMP2 expression in H295R cells following Overexpression of BMP3b
H295R cells were transfected with an empty vector (pIRES) or a vector expressing BMP3b (pIRES/BMP3B). BMP2 expression was measured using real time PCR. * p<0.05
Graph taken from work by Dr A Bakmanidis.

1.9 Adrenocortical tumours and BMPs

1.9.1 Benign adrenal adenomas

Adrenocortical tumours can be benign or malignant. Benign tumours, especially adenomas that secrete aldosterone are much more common than malignant tumours.

Primary aldosteronism (PA) is a condition where patients classically present with hypertension and associated hypokalaemia. However, hypokalaemia is found less frequently with increased screening or following the incidental finding of adrenal nodules on imaging (Sze et al., 2014). Other symptoms can include headaches, fatigue, muscle weakness, numbness or paralysis: PA thus encompasses a whole array of diagnoses of which some can be cured surgically. The prevalence is thought to be around 10% of hypertensive patients (Douma et al., 2008; Fardella et al., 2000; Lim and Morad, 2004; Rossi et al., 2006). Although benign, it is vital to identify these patients early as treatment could potentially be curative or medically controlled to reduce cardiovascular morbidity and mortality (Abad-Cardiel et al., 2013; Bunda et al., 2007; Reincke et al., 2012; Young, 2008). Conn's syndrome is PA with an aldosterone producing adenoma (APA) and this type of PA is potentially curable with surgery (Mattsson and Young, 2006). Bilateral adrenal hyperplasia (BAH) does not have a distinct mass but the whole adrenal gland is involved and these patients will respond well to aldosterone receptor antagonist treatment such as spironolactone or eplerenone (Funder et al., 2008; Handler, 2012). APA and BAH account for the majority of PA cases. The much rarer causes include the familial forms of PA. Type 1, also known as glucocorticoid remedial aldosteronism (GRA) follows an autosomal dominant pattern and accounts for <1% of PA cases (Chao et al., 2013; Moraitis and Stratakis, 2011). In patients with GRA, there is abnormal fusion of *CYP11B1* and *CYP11B2* genes which are located in close proximity to each other on chromosome 8, making the gene responsive to ACTH. As a result, when ACTH is secreted, this abnormal gene is activated to produce aldosterone synthase, leading to excess aldosterone production (Lifton et al., 1992; Pizzolo et al., 2005). GRA is usually diagnosed in young patients (<20 yr) with severe and treatment resistant hypertension or a haemorrhagic stroke, though some may present with milder phenotypes (Gates et al., 1996). Treatment is with glucocorticoids to suppress ACTH production (Ulick et al., 1990). Type 2 is also autosomal dominant, occurring in families and

reported to account for <7% of PA cases. These families may have adrenal glands which harbour APAs or BAHs (Funder, 2012; Stowasser and Gordon, 2003). The newest type, Type 3 can be sporadic or familial and results in a somatic mutation of the *KCNJ5* gene. This gene, found on chromosome 11q24 encodes an inwardly rectifying potassium channel *GIRK4.* Mutation of this gene leads to reduced selectivity of the potassium channel, allowing the entry of sodium ions into adrenal cells. This excess sodium influx affects cell membrane potential and activates the calcium/calmodulin pathway increasing aldosterone production (Choi et al., 2011).

Since the discovery of *KCNJ5*, there has been an explosion of interest in the genetics of APAs with the identification of *ATP1A1, ATP2B3* and *CACNA1D*. These are all somatic mutations affecting the ion channels (Na^+, K^+ and Ca^{2+}), which have a strong biological effect in the zG. *ATP1A1* is a gene that codes for the α1 subunit of the Na^+/K^+-ATPase, *ATP2B3* codes for the plasma membrane Ca^{2+}-ATPase, type 3, and *CACNA1D* encodes the Cav1.3 channel (calcium channel, voltage dependent, L type, α-1d subunit). *KCNJ5* and *ATP1A1* mutations has an effect on the membrane potential of zG through increased K^+, leading to increased sodium and hence causing chronic depolarisation of the cell membrane, resulting in the opening of voltage-dependent calcium channels. This increased calcium flux will activate the Ca^{2+} signalling pathway and upregulate *CYP11B2* expression and hence increase aldosterone biosynthesis (Boulkroun et al., 2012; Choi et al., 2011; Williams et al., 2015). Both *ATP2B3* and *CACNA1D* mutations increase intracellular calcium concentration leading to increased aldosterone production. *CACNA1D* mutations alter segments bordering the channel pore, resulting in channel activation and opening at a lower voltage (Azizan et al., 2013; Fernandes-Rosa et al., 2014). A large genetic study was carried out by Fernandes-Rosa *et al.,* using samples collected in the European Network for the Study of Adrenal Tumors (ENSAT), they found that 54.2% of APAs had a somatic mutation. Of those, *KCNJ5* was by far the common mutation identified accounting for 38% of cases, similar to other studies indicating its prevalence at around 40% (Azizan et al., 2012; Monticone et al., 2012). This percentage varies depending on ethnic background, as in South East Asia cohorts, the proportion of *KCNJ5* mutations range from 65-77%, with a predominance of female patients (Kitamoto et al., 2015; Yamada et al., 2012; Zheng et al., 2015). In the European cohort, 9.3% had *CACNA1D*, 5.3% *ATP1A1* and 1.7%

ATP2B3 mutations. Each of these genes had mutations on various sites. *KCJN5* had mutations in p.Gly151Arg (c.451G>A or c.415G>C) and p.Leu168Arg (c503T>G). *CACNA1D* had multiple mutations with the commonest being p.Gly430Arg (c.1207G>C), p.Phe747Leu (c.2239T>C) and p.Val1338Met (c.2248A>T). The most prevalent mutation in *ATP1A1* is p.Leu104Arg (c.311T>G). The two most common *ATP2B3* mutations found were p.Leu425_Val426del (c.1272-1277delGCTGGT) or pVal426_Val427del (c.1276-c.1281delGTCGTG) (Fernandes-Rosa et al., 2014). Attempts have been made to correlate the genetic mutation to the morphology of the adrenal cortex and the phenotype of patients. No genotype correlation was found with the zG or zF cellular make up of the adenomas (Fernandes-Rosa et al., 2014), though others have reported a more zF like phenotype with a *KCNJ5* mutation and more zG like in *CACNA1D* and *ATP1A1* mutations (Azizan et al., 2013, 2012). In most published cohorts, *KCNJ5* mutations are more common in younger females (Boulkroun et al., 2012; Lenzini et al., 2015). It was also reported by Fernandes-Rosa *et al.,* that patients with a *CACNAD1* mutation have smaller adenomas, whereas other studies have shown *KCNJ5* mutations have larger adenomas (Azizan et al., 2012; Fernandes-Rosa et al., 2014; Zheng et al., 2015). However, there was no difference in the level of aldosterone production in the Fernandes-Rosa cohort whereas the other cohort did demonstrate a higher aldosterone level.

Another somatic mutation found in APA is *CTNNB1*, which encodes β-catenin. Åkerström *et al.,* found 5.1% of their 198 APAs had a *CTNNB1* mutation. This mutation alters serine/threonine residues in the GSK3β binding domain in exon 3. The mutation prevents phosphorylation, and thus degradation of β-catenin, resulting in aberrant upregulation of Wnt signalling (Akerström et al., 2016; Azizan et al., 2013; Tadjine et al., 2008). More recently, there has been interest in ACTH/MC2R as the driver of PA (El Ghorayeb et al., 2016; Funder, 2016).

1.9.2 Malignant adrenocortical carcinoma

The incidence of adrenocortical carcinoma (ACC) is estimated to be between 0.5 and 2.0 cases per million population. Most patients are diagnosed at stage II and beyond and the 5 year survival drops from 66% in stage I disease to 0% in stage IV (Icard et al., 2001; Sidhu et al.,

2004). The pathogenesis of adrenal tumours is not fully understood and treatment options are limited and rarely curative. Gene mutations, such as in *IGF II*, leads to an overexpression of *IGF II* in ACC (Ilvesmaki et al., 1993). β-catenin is another key player emerging in ACC. Its relationship with tumourigenesis was first documented in familial adenomatous polyposis coli (APC), which result from mutations in the *APC* gene. It is now clear that *APC* mutations will also cause an overactivation of the Wnt pathway, leading to ACC (Blaker et al., 2004). *TP53* is a well-established gene associated with childhood adrenocortical carcinoma in South Brazil, where the incidence of ACC is ten times that of the world population (Figueiredo et al., 2006). It is a p53 germline mutation where there is an arginine to histidine mutation at codon 337 of the *TP53* gene with a penetrance of 9.9%. Childhood ACC are thought to be embryological in origin and one of the genes thought to be crucial in adrenal development, *SF-1,* is overexpressed in childhood ACC (Figueiredo et al., 2005). Other ACC genes investigated include *MEN1, VEGF, FGF2* and *Ras* (Libe et al., 2007). Adrenal tumours are present in 26-45% of MEN1 cases (Barzon et al., 2001; Burgess et al., 1996; Gibril et al., 2004; Langer et al., 2002; Skogseid et al., 1992, 1995). *MEN1* is a gene with an autosomal dominant mutation in exon 2 or 10. The majority of patients have non-functioning or hyperplastic adrenal nodules but 2.6-6% do present with ACC (Griniatsos et al., 2011). Both *VEGF* and *FGF2* are growth factors that are overexpressed in ACC. *FGF2* is expressed in the adrenal gland and is a potent mitogen. *VEGF* upregulation would increase angiogenesis and support tumourigenesis. The involvement of *Ras* oncogene in human ACC is still unclear with no convincing evidence currently (Libe et al., 2007).

1.10 BMPs in adrenal tumourogenesis

The role of BMPs in adrenal tumourigenesis remains under investigation by various groups. BMP pathways have been linked to various different cancers (Johnsen and Beuschlein, 2010). Mutations in *SMAD 4* or *BMPR1A* is associated with an increased risk of colon cancer (Howe et al., 1998; Zhou et al., 2001). Depending on cell type, receptor presence and receptor affinity, BMPs appear to exert different responses and hence the over- or under-expression of BMPs can occur in different cancers. *BMP2* and *BMP3* are down regulated in colon and prostate cancer (Horvath et al., 2004; Loh et al., 2008) but *BMP2* and *BMP4* are upregulated in lung

and colorectal cancer respectively (Langenfeld et al., 2003; Nosho et al., 2005). *BMP4* is also upregulated in hepatocellular and ovarian cancer (Kallioniemi, 2012) and also plays a role in reducing tumour growth in human glioblastomas (Piccirillo et al., 2006).

Within the adrenal specifically, Beuschlein's group in Germany found a downregulation of *BMP2* and *BMP5* in adrenocortical carcinomas (Johnsen et al., 2009). Using ACC cell lines, they demonstrated that there was reduced activity of Smad 1, 5 and 8 along with the reduced expression of *BMP2* and *BMP5*. On treating these cells with BMP2 or BMP5, there appeared to be a dose and time dependent reduction in cell proliferation and viability, indicating that they have a suppressive effect on tumour growth.

1.11 Hypothesis and Aims

Understanding adrenal zonation and its impact on steroidogenesis is extremely important to understand adrenal function in health and disease. This understanding could result in the development of biomarkers and treatments for adrenal diseases, especially in adrenocortical carcinomas where survival remains poor despite advances in medicine. This could also lead to targeted therapy of the signalling pathway or even targeted gene therapy.

In cases of adrenal insufficiency, increased morbidity and mortality exists despite hormonal replacement with glucocorticoids. By understanding the process of stem cell differentiation and gland maintenance, future treatments could aim to direct pluripotent stem cells to become steroidogenic cells hence providing a renewed adrenal gland.

The hypothesis for this thesis is:

BMP3b and *BMP2* play an important role in adrenal zonation and steroidogenesis.

The aims of this thesis are:

1. To demonstrate that *BMP3b* and *BMP2* expression are present in the adrenal gland and to elucidate their effect on steroidogenesis in H295R cells
2. To localise the expression of *Bmp3b*, *BMP2* and its receptors in rodent adrenal glands
3. To study the effect of Bmp3b and Bmp2 on steroidogenesis in cells and rodent adrenal glands
4. To investigate the role of *BMP3b* and *BMP2* expression in adrenal adenomas and carcinomas

2 Materials and Methods

Laboratory reagents were supplied by Sigma-Aldrich (Poole, UK) unless otherwise stated. Chemicals were all certified as analytical grade. Plasticware was purchased from Greiner Bio-one Ltd (Gloucestershire, UK) unless otherwise stated. Ultra pure water (dH_2O) was obtained using the Purite Select Analyst HP water purifier, and used when making all solutions. Where sterility was required, solutions were autoclaved using a Priorclave TACTROL2 or filtered through a 0.2 μm membrane (Nalgene).

2.1 General Buffers and solutions

PBS - 0.2g/L KCl, 0.2g/L KH_2PO_4 (anhydrous), 8.0g/L NaCl, 1.15g/L Na_2HPO_4 (anhydrous), pH 7.4 (Sigma)

Phenol - Add 10ml of equilibration buffer (10mM Tris pH 10 - Sigma) to 100ml of equilibrated phenol (pH6.7) (Sigma) to adjust to pH 7.9. Add 8-Hydroxyquinoline to final concentration of 0.1% (v/v). Store in the dark at 4^{o}C.

3M Sodium Acetate - Dissolve 40.8g of sodium acetate.$3H_2O$ in 80ml of H_2O. Adjust to pH5.2 with glacial acetic acid, make up to a final volume of 100 ml and autoclave.

TE - 10mM Tris pH 8.0, 1mM Ethylenediaminetetraacetic acid (EDTA)

TAE (50x) - 2M Tris, 1M acetic acid and 50mM Na_2EDTA (pH 8.3 at 1x conc.)

6X loading dye - 0.5mM EDTA, 40% sucrose (v/v), 0.25% (v/v) Orange G.

RNAase free dH_2O - Diethyl pyrocarbonate (DEPC) was added to dH_2O to a final concentration of 0.1 % (v/v) and incubated overnight in a fume cupboard prior to autoclaving.

Ethidium bromide - Dissolve 0.5g ethidium bromide in 50ml of 0.1M EDTA solution and store at RT in a light sealed container.

dNTPs - 100 mM dNTPs (Promega) were combined in a 1:1:1:1 ratio of dATP:dCTP:dGTP:dTTP and diluted in dH_2O to make a 10 mM stock solution which was stored at –20 °C.

Tris buffers – Solutions of 1 M Tris-OH and 1 M Tris-HCl were prepared. To prepare a solution of Tris buffer at a particular pH, the two solutions were mixed at the appropriate ratio as shown in Table 2.1and pH confirmed using a calibrated pH meter.

pH	1M Tris-HCl	1M Tris-OH
6.8	969	31
7.2	889	111
7.3	867	133
7.4	837	163
7.5	804	196
7.6	767	233
7.7	724	276
7.8	673	327
7.9	618	382
8	562	438
8.1	509	491
8.2	448	552
8.3	389	611
8.4	334	666
8.5	208	792
8.6	232	768
8.7	190	810
8.8	156	844
8.9	122	878
9	96	904

Table 2.1 Ratios of Tris-HCL to Tris-OH for required pH

2.2 Cell Culture

All work involving the culture of cells was carried out in a class II sterile cell culture hood. Hoods were regularly cleaned with Virkon (Antec International, Sudbury, UK) and with 70 % (v/v) ethanol before and after each use. All sterile plasticware for cell culture was purchased from Greiner unless otherwise stated. Other laboratory plasticware and media were autoclaved prior to use and opened in the cell culture hood. Gloves were worn at all times during cell culture procedures. All cells were incubated in a humidified Sanyo MCO-17A/C incubator (Jencons-PLS, East Grinstead, UK) at 37 °C and 5% CO_2.

2.2.1 Adrenal cell line H295R

NCI-H295R (H295R, ATCC number: CRL-2128) is a human adrenal cortical cell line adapted from the original NCI H295 cells (Gazdar et al., 1990). H295R cells were grown as a monolayer with media made from 1:1 mix of DMEM:F12, 10 ml/L Penicillin/Streptomycin solution [10000 u penicillin and 10 mg streptomycin per ml in 0.9% (w/v) NaCl], 2% (v/v) Ultroser (Biosepra), 1% (v/v) ITS [containing 1mg/ml insulin, 0.55mg/ml transferrin and 0.5μg/ml sodium selenite], at 37ºC in 95% air/5% CO_2 incubators (Cobb et al., 1996).

2.2.2 Maintenance and sub-culture of H295R cells

Solutions:

- Dulbecco's Modified Eagle's Medium (DMEM) – 4500 mg/L glucose, 0.584 g/L L-Glutamine, 3.7 g/L $NaHCO_3$ (Sigma #D5796)
- F12 nutrient media (Ham) – 1802 mg/L glucose, 0.146 g/L l-glutamine, 1.18 g/L $NaHCO_3$ (Gibco, Paisley, UK)
- Penicillin/Streptomycin – formulated to contain 10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% (w/v) sodium chloride (Sigma)
- ITS (100 x) – 1 mg/ml insulin, 0.55 mg/ml human transferrin, 0.5 □g/ml sodium selenite (Sigma #I3146)
- Trypsin-EDTA 1x Solution (T/E) – 0.5 g/L of trypsin and 0.2 g/L EDTA.4Na in Hanks' balanced salt solution (Gibco).

2.2.3 Trypsinisation and passaging

After removal of media from the wells or flasks, trypsin-EDTA (TE) (0.5g/l trypsin, 0.2g/l Ethylenediaminetetraacetic acid (Invitrogen) was added (3mls of TE for a 10cm plate) for 30 seconds and then removed and cells were left for 1-2 minutes with some gentle tapping to allow the cells to fully detach.

When cells reached 80-90% confluency, they were split into new plates. Warm media was added to the cells, the solution was pipetted up and down a few times to break up cell clumps, aliquots were pipetted into new plates and topped up with new media. Based on the rate of cell

growth, the cells were split every 3-4 days in a 1-to-3 dilution. The H295R cell passage number used was between 5 and 15.

2.2.4 Cell freezing for storage and thawing for use

Cells not utilised were frozen for long term storage. The media was aspirated, cells trypsinised and with 2 ml of new media added, cells were transferred to a 15 ml polypropylene tube. This was centrifuged at 100 g for 5mins and the supernatant was removed, leaving the cell pellet at the bottom. The pellet was re-suspended in a freezing solution containing 10% DMSO (Dimethyl Sulphoxide) and 90% FBS (fetal bovine serum). Subsequently, 1 ml aliquots were pipetted into cryotubes (Nalgene), wrapped with tissue paper (to ensure a slow freezing process) and placed in a -80 ^{o}C freezer overnight to achieve a cooling rate of 1 ^{o}C/min. The tubes were placed into a liquid nitrogen tank the following day for long term storage.

To thaw the cells for use, they were removed from liquid nitrogen storage and quickly thawed at room temperature and transferred to a 15ml tube where warm medium was added and spun for 5 minutes at 100g. The medium was then removed and the cells were resuspended in new medium and plated, with change of medium the next day, after cell adherence, to remove any traces of DMSO.

2.2.5 Cell counting – Haemocytometer

Cells were trypsinised and a 50µl sample of cells was applied to the edge of the coverslip with a pipette. The medium was sucked in by a capillary mechanism to fill the chamber, taking care to allow the chamber to fill itself in order to avoid overloading. 4 large grids in the corners were used, with each grid containing 16 smaller squares. Cells in each small square (as represented by the green square in Figure 2.1) were counted using the Leica DMIL light microscope set with a 10x objective. If a cell lied over the grid lines, only the ones lying over the left and bottom lines were counted. If there are too many cells to count accurately, the cell stock was diluted and recounted with a new sample. The total number of cells counted in the 4 big squares was divided by 4 and that number was multiplied by 10^4 to give the number of cells per ml of medium.

Calculation used to derive cell number:
No. of cells counted ÷ 4 = no. cells/100nl

No. cells/100nl x 10^4 = no. cells/ml

No. cells/ml x ml of media in which the cells were diluted = total no. of cells

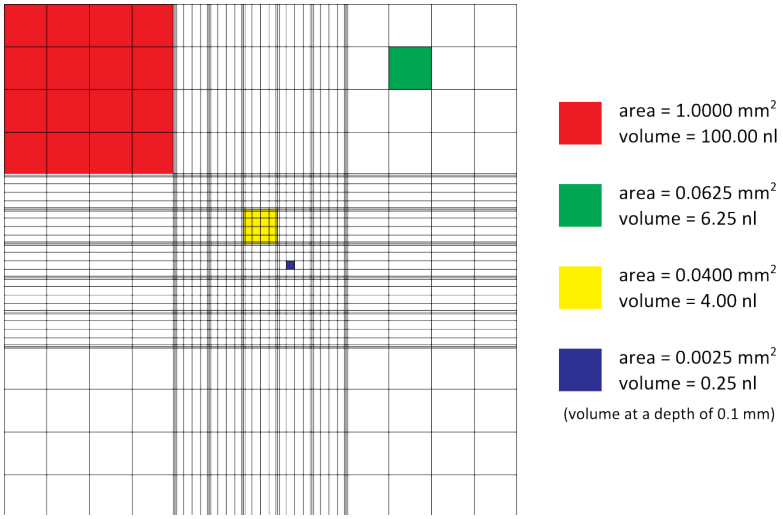

Figure 2.1 Haemocytometer grid as seen under a microscope
Grid demonstrating the volume of each square is shown in the colour key above. Taken from Dr. Katy Cogger (from the King laboratory) with permission.

2.2.6 Cell Differentiation

H295R cells were plated in a 6-well plate (2ml of medium per well) and grown to 50-60% confluency. They were differentiated over 96 hours with fresh media containing 10μM Angiotensin II (Ang II) or 10μM Forskolin in the presence and absence of BMP proteins. The media was changed at 0, 24, 48 and 72 hour time points. At 96 hours, cells were harvested and cDNA was made from total RNA by reverse transcription. Addition of Ang II increases intracellular calcium levels via PKC signalling leading to induction of *CYP11B2* expression, and hence increases aldosterone production (Bird et al., 1993; Rainey et al., 1994). Forskolin activates adenylate cyclase to increase cAMP levels, thus activating PKA, and preferentially inducing *CYP11B1* expression and cortisol production, acutely, and DHEA chronically (Denner et al., 1996; Oskarsson et al., 2006).

To investigate the effect of BMP3b on influencing the steroidogenic differentiation of H295R, leading to up or down regulation of *CYP11B1* and *CYP11B2*, recombinant BMP3b protein (Abnova) at a concentration of 100ng/ml was used. For the experiments investigating the effect of BMP2 and BMP3b on H295R cells towards a zR phenotype differentiation *CYTB5* and *SULT2A1* were used as markers of zR. H295R cells were treated with or without BMP3b or BMP2 (Shenandoah Biotechnology) recombinant proteins at 100ng/ml in fresh media, changed daily over 96 hours and harvested.

2.3 Transfections

Lipofectamine 2000 (Invitrogen) was diluted with optimem I (Invitrogen), and incubated at room temperature for 5 minutes. The DNA or RNAi was also diluted with optimem I then combined with the lipofectamine, vortexed, and left at room temperature for 20 minutes. Cells were then given fresh media and 100µl of the lipofectamine/DNA or lipofectamine/RNAi mix. The media was changed every 24 hours for 96 hours.

2.3.1 siRNA Transfections

Pre-designed BMP3b siRNA (Ambion) at a concentration of 40pmol was transiently transfected into H295R cells using the Lipofectamine 2000 reagent (Invitrogen). Cells were grown in a 12-well plate until they reached 40-50% confluency. Lipofectamine 2000 was diluted with Optimem I (Invitrogen) and left at room temperature for 5 minutes. siRNA was diluted with Optimem I in another tube and added to the Lipofectamine, vortexed and left to stand at room temperature for 20 minutes. Fresh media with Ang II (10µM), Forskolin (10µM) or normal medium (Untreated) was added to the cells, along with 100µl lipofectamine/siRNA. Media and lipofectamine/siRNA was changed every 24 hours for 72 hours and harvested for RNA. The gene expression was analysed by Real-Time Quantitative PCR (qPCR).

2.4 RNA and DNA preparation

2.4.1 Agarose gel electrophoresis

This method was used to estimate the sizes of PCR products when compared to a DNA ladder of known fragment size. Negatively charged DNA was separated on 1-2% (w/v) agarose gel matrix (smaller fragments travel faster), containing GelRed, resulting in the visualisation of distinct DNA bands when examined under UV light. The gel was prepared by melting agarose powder in 1xTAE buffer (1% (w/v) agarose for fragments >500bp, 2% (w/v) agarose for fragments <500bp). Then GelRed (5µl/100ml TAE) was added and the mixture left to set in a mould containing a comb (which would form the wells). The set gel was placed into the electrophoresis tank and covered with 1xTAE buffer. The samples were loaded into the wells, using 10x Orange G loading dye (4g sucrose dissolved in 10mls 1mM EDTA pH8.0 + 20mg Orange G) and run at 110V next to a GeneRuler^{TM} DNA Ladder mix. The gel was viewed on a UV-light box.

Human primers	Tm (°C)	Annealing Temp (°C)	Sequence (from 5')	Product length (Bps)
GAPDH F	62.6	55	TGCACCACCAACTGCTTAG	177
GAPDH R	63		GGATGCAGGGATGATGTTC	
BMP2 F	60.4	53	CTACATGCTAGACCTGTATCG	385
BMP2 R	69.3		ATTTCGAGTTGGCTGTTGCAG	
BMP3b F	64	55	CACTTCTACTCAGAGCCGCC	297
BMP3b R	63.9		GGTCCCTCTCCTCAGAATCC	
BMP4 F	63.6	56	CTTTACCGGCTTCAGTCTGG	235
BMP4 R	63.2		AGATCACCTCGTTCTCAGGG	

BMP5 F	63.9	60	GAGCTGCTGGGTTCTAGTGG	156
BMP5 R	64		AATGGTCTGGGTCTGTGAGG	
BMP6 F	64	51	TGTGAACCTGGTGGAGTACG	233
BMP6 R	64.1		GGGTGTCCAACAAAAACAGG	
Pref-1 F	63.8	58	TGGCTTCTCAGGCAATTTCT	231
Pref-1 R	64.2		GGCTTGCACAGACACTCGTA	
Ptch F	64	56	TGTCACAGCCTTCTTCATGG	241
Ptch R	64.3		GTCGTGTGTGTCGGTGTAGG	
SHH F	64.9	58	ACATCACCACGTCTGACCG	218
SHH R	65.1		GCTCAGGTCCTTCACCAGC	
Gli1 F	63.7	60	CTACAGTGGAGCCCAAGAGG	209
Gli1 R	64.2		GGAGAGGTCTTCAGTGCTGC	
CYP11B1 F	66.5	60	TCCCACACTAATGGCCTAGC	206
CYP11B1 R	66.3		AGCCAGATTTAGGGAGTGGG	
CYP11B2 F			CATACAATGGGTTTGGAGGG	210
CYP11B2 R	65.5	60	TGTCAGATCCCCTAGCTTGC	
CytB5 F	63.7	57	AGTATGTCCGGTTCAATGGC	199
CytB5 R	61.1		GAACCTCTTCTCCTCCAGGG	
SULT2A1 F	64.3	57	AGTGAAACGGAGAGTCCACG	220
SULT2A1 R	64.6		ATGTGGTCAAACCATGACCC	

Table 2.2 List of primers used in RT-PCR and RT-qPCR

2.4.2 RNA Extraction

RNA was extracted from both tissues and the cytoplasm of cultured cells using the Qiagen RNeasy® Mini Kit, as per protocol. RNA was extracted from human tissues that had been collected and stored in RNALater at -80 ^{o}C and approximately 20mg of tissue was homogenised with 600µl of RLT buffer in 2ml Precellys 24 tubes (cat. No. 03961-1-003) and homogenised using the Precellys 24 homogeniser (Bertin) for 3 cycles of 15 second agitations. The supernatant was collected after centrifuging at 12,000g for 3 minutes.

RNA was extracted from cultured cells according to the protocol provided with the Qiagen RNeasy® Mini Kit. The cells were trypsinised with Trypsin-EDTA, washed with cold PBS (Phosphate Buffered Saline) detached with a cell scraper and transferred to 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 12,000 g for 15 seconds, and following removal of the supernatant, 350 µl of RLT buffer with the addition of β-mercaptoethanol was added to the cell pellet. If cells were grown in smaller wells (12 well plates or smaller), following the cold PBS wash, the cells were lysed directly in the wells with 350 µl of RLT buffer + β-mercaptoethanol. The lysates were then transferred to 1.5ml microfuge tubes using a cell scraper. The RNA concentration was determined using the Nanodrop ND-1000 spectrophotometer to measure the optical density of the samples at 260 nm wavelength (OD_{260}). An OD_{260} of 1 corresponds to approximately 50 µg/ml for double stranded DNA and 40µg/ml for single stranded DNA and RNA. 2µg of RNA was used to make cDNA, based on the following calculation:

OD_{260} x f x d = concentration of nucleic acid (µg/ml)

Where: OD_{260} = optical density of the sample at 1 cm path length, f = 40 for RNA or 50 for DNA, d = dilution factor of sample

2.4.3 cDNA Preparation with DNase treatment

This process cleaves and fragments any genomic DNA contamination. This was achieved with DNaseI (Deoxyribonclease I), which non-specifically cleaves single- and double- stranded DNA and RNase inhibitor 40u/µl (Promega), which reduces any RNase activity, hence preventing RNA degradation.

A single reaction contains:

2µg RNA	=> Xµl
RNase Free H_2O (Qiagen)	=> 43.5µl-Xµl
10x DNase Turbo buffer (Ambion)	=> 5µl
DNase 1	=> 1µl
RNase inhibitor	=> 0.5µl

TOTAL	=> 50µl

The reaction was vortexed briefly and incubated in a water bath at 37ºC for 15mins.

2.4.4 Phenol extraction and precipitation:

This step uses the organic solvent, phenol, equilibrated to pH8.0, to denature and remove protein contaminants, genomic DNA and enzymes from the RNA.

An equal volume of phenol was then added to stop the reaction. Each reaction was vortexed for 1 minute and centrifuged at 10000 g for 2mins. The reaction then separates into 2 phases: the top aqueous phase, containing RNA, is removed and placed into a new microfuge tube and the lower phenol phase on the bottom containing the organic and inter-phases which is discarded.

2.4.4.1 RNA precipitation:

RNA precipitation allows any residual phenol to be removed:

RNA (x)	=> 50µl
3M Sodium Acetate pH 5.3 (x/10)	=> 5µl
Glycogen (inert carrier 5mg/ml, Ambion)	=> 2µl
Absolute Ethanol (2.5x)	=> 125µl

The reaction was vortexed for 30 seconds and left at -80 ºC for 1 hour or -20 ºC overnight. The sample was then centrifuged at 10000 g for 10 minutes at 4 ºC. The supernatant was aspirated

and the precipitant (white pellet) washed with 125 µl 70% (v/v) ethanol, to remove any precipitated sodium acetate. Samples were centrifuged for 5 mins and the ethanol was aspirated. The final pellet was air-dried for 10 minutes.

2.4.4.2 First strand cDNA synthesis:

The air dried pellet was resuspended in 12.25 µl of RNase free H_2O and 0.25µl of random primers (500 µg/ml) (Promega). The sample was heated for 10 minutes at 80ºC to denature the secondary structure in the RNA and immediately placed on ice for 2 minutes to allow the random primers to anneal.

The sample was quickly centrifuged to pool any condensation and a 1 µl aliquot was removed to be used for RT-PCR negative controls (these should not contain any traces of genomic DNA) and the remainder used for cDNA synthesis.

A single cDNA synthesis reaction contains:

2 µg RNA /Random primers sample	=> 11.5µl
5x Reverse Transcription Buffer (Promega)	=> 4µl
DTT (Dithiothreitol, 0.1M)	=> 2µl
dNTPs (deoxynucleoside triphosphates, 10mM A+C+G+T; Promega)	=> 1µl
M-MLV Reverse Transcriptase 200u/µl (Promega)	=> 1µl
RNase inhibitor 40u/µl (Promega)	=> 0.5µl

TOTAL	=> 20µl

The reaction was then vortexed and centrifuged briefly and then incubated at 37ºC for 1hour for the reverse transcription to occur. After incubation, 20µl distilled water was added to the newly synthesised cDNA, giving a 1 in 2 dilution. Successful cDNA synthesis is verified by

RT-PCR with GAPDH followed by 1% (w/v) agarose gel electrophoresis of samples with negative control (water) (Figure 2.2). cDNA was stored at -20^{o}C.

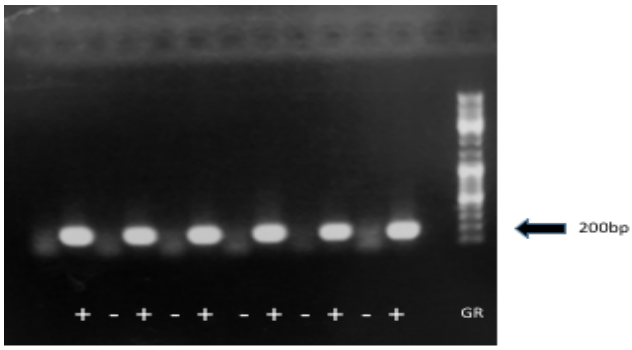

Figure 2.2 Agarose gel (1%) electrophoresis to demonstrate successful cDNA synthesis using GAPDH primers
+ = newly synthesized DNA, - = water as negative control, GR = GeneRuler, bp = base pair

2.4.5 Silica gel extraction

DNA was extracted from the Glass Milk gel extraction technique. The desired bands were excised from the gel using a clean scalpel. The excised gel bands were melted at 55^{o}C for 5-10min (until gel has totally melted) in 3 volumes of 6M NaI for every 1 volume of gel. 10µl of the silica suspension was added and incubated at room temperature for 5 mins, flicking intermittently to keep silica in suspension. The suspension was centrifuged for 15 sec at 10000g and the supernatant aspirated. The pellet was then re-suspended in 500µl of New Wash buffer (50mM NaCl, 10mM TrisHCl pH7.5, 2.5mM EDTA, 50% (v/v) ethanol). This was repeated twice and centrifuged again to give a dry pellet. The pellet was then resuspended in 10 µl of dH_2O and incubated at 55^{o}C for 2mins. The suspension was finally centrifuged at 10,000 g for 1 minute and the supernatant, containing the DNA, transferred to a new centrifuge tube and stored at -20 ^{o}C

The silica suspension (100mg/ml) was prepared by mixing 10g of silica in 100ml PBS and the silica was allowed to settle for 2 hours. The supernatant was removed and the process repeated. The suspension was centrifuged at 2000g for 2mins; the silica pellet was resuspended in 3M NaI at 100mg/ml and used as a DNA-binding matrix (stored at 4^{o}C in the dark).

2.5 Polymerase Chain Reaction (PCR)

The analysis of cDNA was carried out by PCR (Polymerase chain reaction) where GAPDH primers were tested against the newly synthesised cDNA and the negative control aliquots (removed at the earlier stage). The reactions were analyzed on a 2% (w/v) agarose gel where GAPDH primers gave clear bands (PCR product) with the cDNA samples but no bands in the negative control lanes. PCR was used to analyse the newly synthesised cDNA. PCR is generally used to amplify small quantities of DNA where Taq DNA polymerase (*Thermus aquaticus,* NEB) is used in a polymerisation reaction to make exact copies of the DNA template.

Intron spanning primers were designed using the "Biology WorkBench version 3.2" software (http://workbench.sdsc.edu) where certain designing parameters were met: GC clamp size of 2, optimum primer size of 20 bases, optimum melting temperature of 60°C, GC content 40-60%. A single PCR reaction contains:

dH_2O	=> 19.375µl
10x PCR Buffer	=> 2.5µl
dNTPs (10mM)	=> 1µl
Primers (10µM For + Rev)	=> 1µl
Taq DNA Polymerase	=> 0.125µl
DNA template	=> 1µl

TOTAL	=> 25µl

Cycles	Temperature	Time	Process
1	94°C	5 minutes	Denaturation
25-35	94°C	30 seconds	Denaturation
25-35	45-65°C	30 seconds	Annealing
25-35	72°C	30 seconds	Extension
1	72°C	10 minutes	Further elongation
	4°C	∞	Preservation

Table 2.3 Cycle and temperature profile used for RT-PCR

Denaturation at 94°C

→ The double stranded DNA melts to single stranded DNA by denaturing hydrogen bonds.

Annealing at 45-65°C

→ The primers bind their matching single stranded sequences; the polymerase then attaches and begins copying the sequence. This temperature is variable depending on the nucleotide content, hence the melting temperature (Tm) of the primers.

Extension at 72°C

→ Optimum polymerisation temperature; Taq polymerase continues to add complementary dNTPs onto the sequence from 5' to 3'. For longer PCR products an extension time of 1 min for every 1000k bp was used.

The PCR products were analysed with agarose gel electrophoresis. Whenever no product was obtained from the PCR reaction, different reaction conditions were tested such as lowering the annealing temperature

2.6 Real-Time Quantitative PCR (SYBRgreen I)

The differences in gene expression were quantified using the SYBRgreen Real-Time qPCR approach. SYBRgreen exhibits greatly increased fluorescence (up to 1000 fold) when it non-specifically binds to double-stranded DNA (dsDNA). The total fluorescence signal is proportional to the total amount of the target dsDNA amplification; hence mRNA levels can be calculated. A value obtained from qPCR is called a Ct value. The Ct value/level represents the point when amplification is first detected above background fluorescence, during the exponential phase of the amplification. The lower the Ct value, the higher the amount of starting DNA present. The initial (starting) gene copy number was calculated by plotting against the standard curve and the gene expression was normalized to GAPDH (Figure 2.3). A standard curve with serial dilutions of gel extracted product of known DNA concentration of each gene was made, so copy numbers were derived from the Ct values. The reference gene GAPDH was used to account for differences in the amount of cDNA loaded. The dissociation curve tests for non-specific amplification and contamination. The dissociation melt separates double stranded DNA back into single strands. Pure products only give one peak at its corresponding melting temperature and the no template control (NTC) should give no peaks or a smaller peak at a different melting temperature to the target product, indicating primer dimers. Intron spanning primers were designed to give products of approximately 200bp and the final primer concentration ranged from 100-200nM (to reduce primer dimers). ROX in the reaction is used as a reference dye for evaporation during cycling.

For the experiment to be valid, the following criteria were met:
Efficiency = 100% (± 10%)
Standard curve gradient = -3.3 (± 0.3)
R^2 = 0.97-1

The KAPA SYBR FAST qPCR kit (Kapa Biosystems) was used. All of the reactions were performed in duplicates or triplicates, using a no-template control (NTC) which consists of water to look at background fluorescence. The cDNAs were plated out onto 96 well plates or

0.2ml strip tubes and centrifuged at 10000g for 2mins to remove any bubbles. The reactions were loaded into the MxPro Real-Time PCR machine (Stratagene).

A single reaction contains:

2x Master Mix => 5 µl
dH_2O => 2.3 µl
Low ROX => 0.2 µl
Primers (F + R) => 0.5 µl
DNA template => 2 µl

TOTAL => 10 µl

Cycles	Temperature	Time	
1	95°C	3 minutes	Denaturation
40	95°C	3 seconds	Denaturation
40	60°C	30 seconds	Annealing
40	72°C	1 second	Extension
1	95°C	1 minute	Dissociation melt
1	60°C	30 seconds	

Table 2.4 Cycle and temperature used for RT-qPCR

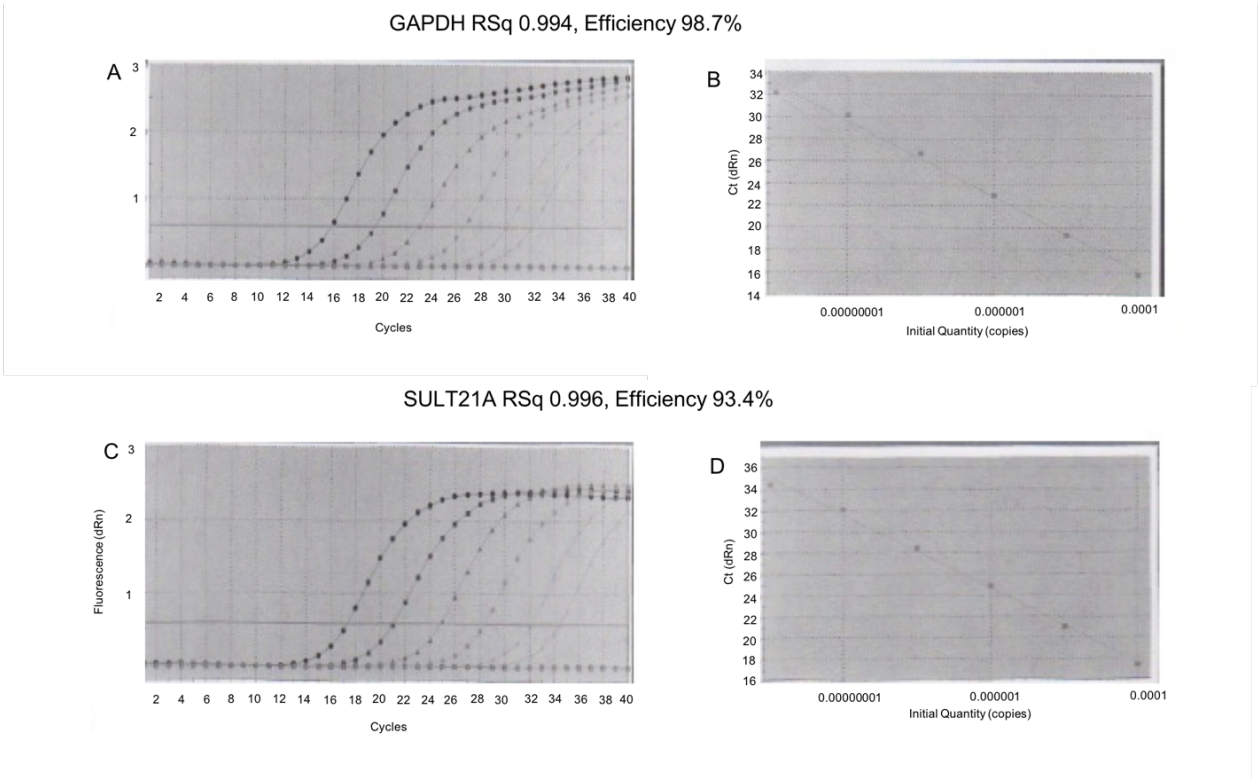

Figure 2.3 qPCR amplification plots and standard curves
Demonstration of amplification plots (A,C) and standard curves (B,D) for qPCR experiment for *GAPDH* (A,B) and *SULT2A1* (C,D) mRNA expression.

2.7 Enzyme-linked immunosorbent assay

H295R cells were transfected with BMP2, BMP3b and siBMP3b and each were treated with vehicle, Forskolin or Ang II. The media was changed every 24 hours (for 3 days with siRNA and 4 days for other recombinant BMPs). The media were stored at -20°C until ELISA carried out. ELISA kits were obtained from Demeditec Diagnostics. The media was tested for cortisol, DHEAS and aldosterone as per instruction provided. The experiment was carried out at room temperature. Calibrator wells were set up and experimental media was tested using 50µl of media per well, in duplicates. The media was centrifuged for 15 seconds at >10,000 x g to pellet any debris, and 25µl of supernatant was added to each well of a Demeditec ELISA microtiter plate. Plates were pre-coated with either an anti- aldosterone, an anti-cortisol or an anti- DHEAS antibody. A standard curve containing known quantities of aldosterone, cortisol

or DHEAS was also plated. 100 µl of conjugate working solution was then added and placed on a shaker plate at 200 rpm for 1 hour at room temperature. Wells were then washed 3 times with 300 µl diluted washer buffer. 150 µl TMB (tetramethylbenzidine) substrate was then added at timed intervals and then incubated for another 20 minutes on the shaker plate. The enzyme reaction was stopped by adding 100 µl of Stop solution and the plate was read on a microwell plate reader at 450nm immediately (within 20 minutes) using the Perkin Elmer Wallac Victor2 1420 Microplate reader. Using the calibrators, a standard curve was drawn to read the values of the experiment, where the concentration of aldosterone, cortisol or DHEAS was determined from the absorbance values from the standard curve.

2.8 Cloning

Primers were designed against the coding sequence of the desired gene, which was amplified by PCR. The product was run on an agarose gel and the expected product size was confirmed against a DNA ladder. The band was cut out of the gel and DNA extracted was either sequenced or confirmed by a restriction digestion. After the sequence was confirmed the gene was thenncorporated into a plasmid.

Primer (Rat)	Tm (°C)	Annealing temp (°C)	Sequence (from 5')	Product length (Bps)
BMP2 F	64.3	58	CCCCTAGTGCTTCTTAGACGG	923
BMP2 R	63		CTGGACTTAAGACGCTTCCG	
BMPR1A F	63.7	57	GTATGGATGGGTAAATGGCG	906
BMPR1A R	63.9		TCCTTGGGTGAATTTCTTGC	
BMPR1B F	64	56	AGACCTCGGTACAGCATTGG	946
BMPR1B R	63.7		TCATAAGCTTCCCCATTTGC	
BMPRII F	63.9	55	ATCCCAATGGATCTCTGTGC	955
BMPRII R	63.8		CTAGGGATTCGAGCTTGTGC	
BMP3b F	59.9	57	AAATGATCCTCACAGCCACC	1024
BMP3b R	60.0		ACCTTCAGAACCACATTCCG	

Primer (human)	Tm (°C)	Annealing temp (°C)	Sequence (from 5')	Product length (Bps)
BMP3b F	63.2	58	GGCCGTGTATTTCTTCAACC	1015
BMP3b R	63.6		ACCCCAAGGGAGTTCATCTT	

Table 2.4 Primers used to produce inserts for in situ hybridisation

2.8.1 Plasmids

The pGEM®-T easy vector (Promega) (Figure 2.4) was used for the cloning. It is already cut and contains 3' terminal thymidine overhangs at both ends; this prevents vector re-circularization and provides a compatible overhang for PCR products. An advantage of using this vector is that it could be subjected to blue-white screening, making it easier to locate successfully cloned colonies.

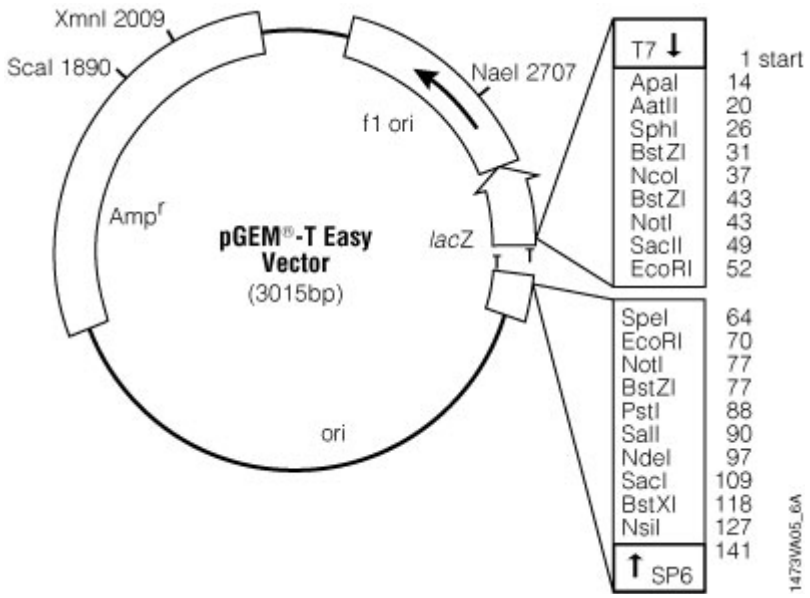

Figure 2.4 pGEM-Teasy vector and restriction map used for ligation

2.8.2 Ligation

The concentration of plasmid and insert DNA was determined by spectrophotometry or compared to the expected weight by comparing with the DNA ladder. A 3:1 (insert:plasmid) ratio was used. All the inserts used were around 1000bp, therefore, based on the insert:vector ratio calculation, 50ng of insert were used for each reaction, along with 50ng/µl of plasmid.

A single ligation reaction contains:

2X Rapid Ligation Buffer	=> 5 µl(x1 final)
Insert	=> X µl(50ng)
Plasmid (pGEMTeasy)	=> 1 µl(50ng)
T4 DNA Ligase	=> 0.5 µl
dH_2O	=> 3.5µl - X µl
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TOTAL	=> 10 µl

The reaction was incubated at room temperature for 3-4 hours. After the ligation was complete, the plasmids were transformed into bacterial cells.

2.8.3 Transformation

The ligated DNA (plasmid + insert) was transformed into competent bacteria which were grown on agar plates under selection to obtain colonies containing the plasmids. Agar plates were prepared by dissolving 10g L-Broth and 7.5g Agar in 500ml dH_2O and autoclaved. The mixture was poured into sterile 10cm petri dishes, and once set, dried upside-down in 37°C bacterial incubators and stored at 4°C (upside-down). For antibiotic selection, 500µl of an Ampicillin stock (100mg/ml) was added to the 500ml of LB solution (100µg/ml final concentration), after it has been cooled to <50°C, before pouring the solution into the dishes. For blue/white screening, 100µl 0.1M IPTG (isopropyl-beta-D-thiogalactopyranoside) and 50µl X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20mg/ml) (Promega) were directly pipetted onto the agar plate and spread until dry (near a Bunsen-burner flame) and incubated at 37°C, until ready to be used. When the insert was successfully ligated into the plasmid, the coding sequence of the lacZ gene is disrupted resulting in the inability of the bacteria to cleave the chromogenic substrate X-Gal to form a blue coloured colony. In theory, all the white colonies should contain the plasmid with insert.

The plasmid was transformed into the JM109 (Promega L2001) competent bacterial cells as per their protocol. Briefly; 2-5 µl of the ligation reaction was gently mixed with the bacterial cells (60-100 µl), incubated on ice for 10 – 30 minutes (depending on which protocol), heat shocked at 42°C for 45 seconds and immediately returned into ice for 2 minutes. The bacteria were incubated with SOC medium for 1 hour at 37°C with shaking at 225 rpm. Subsequently, the bacteria were centrifuged for 5 minutes at 21°C, at 200 g. 800µl of the supernatant were removed and 50 µl of the transformation was plated onto one plate and about 150 µl onto a second plate. Plates were incubated upside-down at 37°C overnight. The grown bacterial colonies were picked from the plate (for blue/white screening the white colonies were picked) and grown to saturation in 5ml of L-Broth with 5µl of Ampicillin [100mg/ml] in a shaking bacterial incubator, at 37°C overnight. The bacteria were used in further steps to isolate the desired plasmid.

2.8.4 Plasmid extraction and purification

The plasmid was extracted from bacterial culture by alkaline lysis. 1.5 mls of bacterial culture was pelleted by centrifugation and the pellet was resuspended by vortexing in the residual broth after removing most of the supernatant (approximately 20 µl). The bacteria were lysed by vortexing in 300µl of a 1:2 mix of solutions 1 and 2and then neutralised with 150 µl solution 3 (Bimboim and Doly, 1979). The resulting precipitated solution was extracted with 400 µl phenol pH8.0 and 400 µl of the aqueous phase was then removed and the nucleic acids precipitated by the addition of 1ml absolute ethanol. After pelleting the DNA by centrifugation at 10000g for 5 mins, the ethanol was aspirated and the pellet washed with 100 µl of 70% (v/v) ethanol. After air-drying for 5 mins, the pellet was resuspended in 50ul TE + 100 µg/ml RNAse A. The plasmid DNA was then further analysed by restriction digestion.

The plasmids were cut with restriction enzymes in order to either linearise them, excise the target gene out of a plasmid or to check if the PCR product insert is correct in size or orientation. The reaction was incubated in a 37^{o}C water bath for 2 hours. A single restriction digestion reaction contains the following:

Note that BSA is not needed with some enzymes, in these cases it was replaced with dH_2O

10X Restriction Buffer	=> 2 µl
DNA	=> X µl (1-2 µg)
Enzyme (10u/µl)	=> 0.5 µl (5u)
BSA (10mg/ml)	=> 2 µl (0.1mg/ml)
dH_2O	=> 15.5 - X µl
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TOTAL	=> 20 µl

To check if the PCR product was correct, the expected target sequence was analysed in NEBcutter v2.0 (http://tools.neb.com/NEBcutter2/index.php) and two restriction enzymes were chosen to cut the product into dissimilar fragments. These fragments were then run on an

agarose gel and if the pattern was comparable to the one expected then the product was thought to be correct (Figure 2.5).

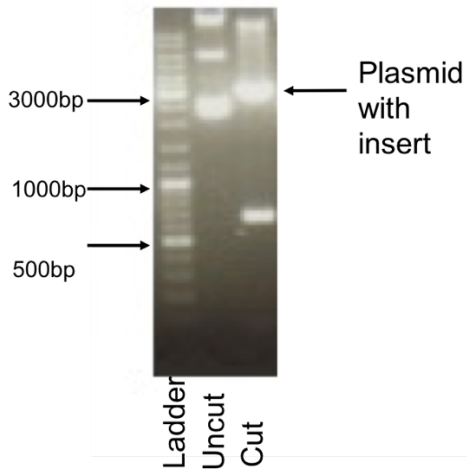


Figure 2.5 Agarose gel showing presence of an insert in the plasmid
Following ligation and transformation, the plasmid is extracted and purified. This is an example of BMP2 after digestion with Nco1. The 'Cut' column includes the plasmid and insert with a combined size of 4106bp.

2.9 Histology

Adrenal glands were excised from male and female Wistar rats from embryological age E14.5, E15.5, E17.5, E19.5 and adults. Adrenal glands were also excised from male and female *Bmp3b* knockout mice. Tissues were either snap frozen and stored in a -80^{o}C freezer or fixed in 4% (w/v) Paraformaldehyde/PBS overnight at 4°C for paraffin or sucrose embedding. Sectioned adrenal tissue were cut to a thickness of about 5-10µm and placed onto Superfrost Plus slides (for fresh frozen and sucrose embedded) and TESPA (3-Aminopropyl Triethoxysilane from Sigma) treated Superfrost slides for the paraffin slides. The slides were used for immunohistochemistry, immunofluorescence or non radioactive in-situ hybridisation to locate gene expression, visualised under a light microscope.

2.9.1 TESPA treated slides:

TESPA (3-triethoxysilylpropylamine) treatment of slides ensures that tissue sections adhere tightly and avoid detachment during subsequent washing procedures in all the staining processes. The advantage of using TESPA is that the treated slides have a long shelf life but tissue sections need to be dried fully before use to ensure tight adherence. The slides were dipped in Acetone and allowed to dry. The slides were then dipped into an Acetone solution containing 2% (v/v) TESPA for 2 minutes. The slides were washed twice in distilled water, 2 minutes per wash and dried overnight at 37^{o}C.

2.9.2 Wax embedding and sectioning

The adrenals were dehydrated through a series of increasing ethanol concentrations, after having been in 4% (w/v) Paraformaldehyde/PBS overnight at 4^{o}C. The volume of washes used was approximately 8x volume of the adrenal. The PBS and water used for dilution for the ethanol concentrations were all DEPC (Diethylpyrocarbonate) treated to prevent RNA degradation by RNases. DEPC inactivates RNase enzymes by covalent modifications of the histidine residues. To DEPC treat water or PBS, 1ml DEPC was added to 1L water or PBS (0.1% v/v), mixed, incubated at 37^{o}C overnight and then autoclaved for 15 minutes to denature the DEPC (which is highly active). In the meantime, the wax was melted in the oven at 56^{o}C. The procedure is listed below:

DEPC PBS for 1hour
DEPC Water for 1hour
DEPC Water for 1 hour
50% v/v Ethanol for 1hour
70% v/v Ethanol overnight
90% v/v Ethanol for 1hour
100% v/v Ethanol for 1hour
100% v/v Ethanol (new solution), overnight at 4^{o}C
Xylene for few seconds – minutes, depending on size of tissue
Repeat Xylene for few seconds – minutes, depending on size of tissue
Molten paraffin overnight at 56^{o}C

The adrenals were placed into a mould, topped up with molten wax, covered and left to solidify overnight at RT. The block of wax was inserted and aligned into a Leitz 1512 microtome, ready for cutting and mounting using the floating out technique as described by Sack (Sack, 1963). The paraffin block was trimmed and paraffin ribbons were cut on the microtome, forming serial tissue sections of 5-7μm in thickness. A small amount of DEPC water was placed onto a TESPA slide and the ribbon placed over the water and heated over a low heat (~ 40-45°C) to gently melt the paraffin to remove any folds within the sections. Once the folds in the section has smoothed out, the water is removed from one corner, sucked up using a plastic 1ml pipette, leaving the sections bonded to the adhesive TESPA coating. Slides were air-dried at room temperature for 30 minutes, then overnight at 37°C and stored at room temperature until use.

2.9.3 Deparaffinisation

For the slides to be used for staining, the paraffin will need to be removed. To deparaffinise and rehydrate the slides, they were washed in xylene 3 times, 100% ethanol twice and once in 90% (v/v), 70% (v/v) and 50% (v/v) ethanol and finally twice in H_2O. Each wash was for 10 minutes.

2.9.4 Cryo embedding

Following the overnight incubation in 4% (w/v) PFA/PBS, the tissue was washed in DEPC PBS. It was then transferred to a 30% (v/v) sucrose/DEPC PBS solution and left at 4°C, upright, in a 50ml Falcon tube until the tissue sank to the bottom. Foil containers were made and placed in dry ice and the tissue placed in the centre, OCT medium (Lamb Laboratories) was added to totally cover the tissue and allowed to freeze at -80°C for at least 20 minutes before use. Tissues were sectioned using a cryotome onto SuperFrost Plus slides and stored at -80°C, if not used immediately.

2.10 Staining

2.10.1 Immunohistochemistry (tissue sections)

The fresh frozen or cryoprotected tissues were cut on a Leica CM 1510 cryostat, set at -20°C and placed onto the positively charged Superfrost slides. The slides were dried off at 37°C and then fixed in ice cold PFA for 15 minutes if fresh frozen or, if sucrose embedded, transferred to the PBS wash step, 3% (v/v) hydrogen peroxide in PBS for 1 hour and then antigen unmasking (see below).

The paraffin adrenal slides were washed three times with xylene, and once with 100% ethanol. Each wash was for 10 minutes. Peroxidase activity was blocked by incubating with a blocking solution made up of 3% (v/v) hydrogen peroxide in methanol for 1 hour. The slides were hydrated through a series of graded ethanol concentrations (10mins in 100%, 90%, 70%, 50% v/v) and then gently washed with dH_2O twice, each 10 minutes. Antigen unmasking was performed by boiling the slides for 30 minutes in 980mls dH_2O with 20ml 0.5M Sodium Citrate, pH6 added. The slides were cooled with a PBS wash and then blocked for 1 hour at room temperature with 10% (v/v) normal serum of the animal the secondary antibody was made in to reduce background staining. The normal serum is made up with PBS + 0.1% (w/v) Sodium azide + 0.2% (v/v) Triton X-100. The edges of the slides were marked with a hydrophobic marker (Pap pen from VWR) and 400µl were used per slide.

For mouse sections, which are known to have an abundance of endogenous biotin, 2 drops of Avidin was added for 15 minutes, followed by a quick PBS/triton (Tr-PBS) wash and then 2 drops of Biotin was added for 15 minutes. Following this (and for rat and human sections), primary antibodies with Tr-PBS were added in the concentrations in Table 2.5 and were left overnight at room temperature. The following day, three 10 minute Tr-PBS washes were done and then the secondary Biotinylated antibody 1:400 concentration was added. The Biotinylated antibody used was raised against immunoglobulins from the animal in which the primary antibody was made. 300µl were used per slide and incubated for 2 hours at room temperature. A further three 10 minute Tr-PBS washes were done and whilst the washes were

on going, the ABC reagents (Avidin + Biotinylated horseradish peroxidise macromolecular Complex) were made up (Vectorlab). Firstly, the total amount of reagents needed was calculated and half of the amount is made with ABC. For example, if the total amount needed was 1000µl, then 10µl of A and 10µl of B was added to 480µl of PBS and incubated for 30 minutes at room temperature before use. This incubation releases peroxidises necessary for the IHC reaction. Just before use, 500µl of PBS was added. 250-300µl were used per slide and incubated for 1 hour at room temperature. Then three 10 minute washes were done and DAB were made. For each 2.5mls H_2O (with a drop of tween added), 1 drop of buffer, 2 drops of DAB and 1 drop of hydrogen peroxide was added in this order. 250µl was added to each slide and incubated for 2-10 minutes, checking for development regularly and once the desired intensity was reached, the reaction was stopped by placing the slide in dH_2O. If there was no staining by 10 minutes, it would be unlikely that any further development will occur beyond this time. The slides then went through a dehydration process of 5 minute washes of H_2O, 50% (v/v), 70% (v/v), 90% (v/v), 2x 100% EtOH and 3x xylene washes. The slides were then mounted using DPX mounting medium and covered with a glass coverslip. Images were acquired using a Leica DMR microscope (Leica, Nussloch, Germany), and digital images were captured using a Leica DC200 camera (Leica) and DCViewer software (Leica). Images were imported into Photoshop (Adobe Systems, San Jose, CA).

2.10.2 Immunofluorescence

For fresh frozen slides, 12 µm sections were cut using the cryotome, placed onto SuperFrost Plus slides and were dried at 37°C (for about 3 hours) then fixed in cold PFA for 15 minutes before going onto 3x 10 minute PBS washes. Sucrose embedded sections were dried and went straight to PBS washes. Paraffin sections were deparaffinised as set out above. For paraffin and sucrose embedded tissue, the sections underwent an antigen unmasking step by boiling the slides in citrate buffer (pH6) for 30-40 minutes and then allowed to cool. This step breaks any protein crosslinking formed by PFA treatment and thus exposes antigenic sites which may have been masked. All sections were blocked for 2 hours at RT with 10% (v/v) normal animal serum, as in IHC and then primary antibody added and incubated at 4°C overnight in the concentrations set out in Table 2.5. The next day, 3x Tr-PBS washes was followed by the addition of secondary antibodies for 3 hours at RT. For a negative control, the primary

antibody was not added to assess the level of non-specific binding. Another slide had only secondary antibody added in order to assess the level of background, to ensure the staining was valid. 3x PBS washes was followed by 1 minute incubation with DAPI (4',6 Diamidino-2-phenylindole dihydrochloride 1:5000 concentration with dH_2O), used to label nuclei. Slides were washed for 3x 10 minutes with PBS and mounted in glycerol: PBS (ratio 4:1) solution and covered with glass coverslip. Images were acquired using a Leica DMR microscope (Leica, Nussloch, Germany), and digital images were captured using a Leica DC200 camera (Leica) and DCViewer software (Leica). Images were imported into Photoshop (Adobe Systems, San Jose, CA).

Antibody		Company	Concentration
CYP11B1	Monoclonal mouse	Prof Celso Gomez-Sanchez	1 in 15
CYP11B2	Polyclonal rabbit	Dr Ed Laufer	1 in 500
Pref 1	Polyclonal rabbit	Prof Gavin Vinson	1 in 400
Side chain cleavage	Polyclonal rabbit	Abcam	1 in 1000
BMP 2	Polyclonal goat	Abcam	1 in 200

Table 2.5 Primary antibodies with concentrations used

Antibody	Company	Fluorescence	Colour	Concentration
Goat anti mouse	Invitrogen	AF488	Green	1 in 1000
Goat anti rabbit	Invitrogen	AF568	Red	1 in 1000
Donkey anti rabbit	Jackson ImmunoResearch	CY2	Green	1 in 1000
Donkey anti goat	Jackson ImmunoResearch	CY3	Red	1 in 1000

Table 2.6 Secondary antibodies with concentrations used

2.10.3 *IN SITU* HYBRIDISATION

RNA was extracted from adult Wistar rat adrenal tissues or H295R cells using the RNeasy Mini kit (QIAGEN), and cDNA was prepared from random-primed total RNA using Maloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT, Promega) following the manufacturer's instructions. See Table 2.4 for gene and primers used. Amplified cDNAs were cloned into the dual promoter vector pGEM-T easy (Promega) and linearised with the appropriate restriction enzymes, as above. Digoxigenin (DIG)-labelled antisense and sense cRNA probes were synthesised by *in vitro* transcription in the presence of DIG-labelling mix (Roche) following the manufacturer's instruction and using 1 µg of linearised template and T7 or SP6 RNA polymerase (New England Biolabs). The concentration and integrity of each RNA probe was analysed by gel electrophoresis and spectrophotometrically (Nanodrop ND-1000); for each probe, the transcription reaction resulted in approximately 10 µg of DIG-labelled RNA, which was diluted with DEPC-treated H_2O to a concentration of 100 ng/µl, aliquoted, and stored at –80°C. All probes were used at a concentration of 400-800 ng/ml in hybridisation buffer. Each probe was designed to include regions of low nucleotide identity with other related family members or other sequences located in the National Center for Biotechnical Information nucleotide database.

Sections were either dehydrated through ascending alcohol series (paraffin) or fixed in PFA (fresh frozen or cryoprotected). They were then all permeabilised with Proteinase K (5 µg/ml in 100mM Tris HCl pH 7.5 and 50mM EDTA, pH 8.0) for 10 min at 37°C. After washes in PBS-0.1% (v/v) Tween-20 (T-PBS), slides were acetylated in T-PBS containing 0.25% (v/v) acetic anhydride and 0.1% (v/v) triethanolamine (pH 8.0) for 10 min at room temperature (RT). Finally, sections were prehybridised at 58°C in a solution containing 0.3 M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA, 50% (v/v) formamide, 5% (w/v) dextran sulfate, 1× Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.02 % (v/v) SDS. After 1 hr 30 minutes of prehybridisation, the probes were added (400 µl per slide, covered by a Parafilm "M" coverslip), and the hybridisation reaction was allowed to proceed for about 17 hr at 58°C. After hybridisation, the sections were washed in decreasing concentrations of saline sodium citrate (SSC) (2X, 1X, 0.2X and 0.05X, where 2X is 0.3M sodium chloride and 0.03M sodium citrate, pH 7.0) at room temperature for 20 min then at 65°C for 15 min at each concentration.

The slides were then washed in STE buffer (0.5M NaCl, 10mM Tris pH 7.5, 5mM EDTA, 0.1% (v/v) Tween) for 10 min at RT followed by treatment with RNase A (25 µL of 20mg/ml stock in 50 ml of STE buffer) for 30 minutes at 37°C. After washing the slides twice with Maleic Acid Buffer (MAB) (0.1M maleic acid, 0.15 NaCl, pH 7.5, stored at 4°C) they were incubated with anti-DIG alkaline phosphatase-Fab fragments (Roche) diluted to 1:2000 with 0.5% (v/v) Blocking Reagent (Roche) in MAB/0.1% (v/v)Tween 20, overnight at 4°C.
After two washes with T-PBS the slides were equilibrated in alkaline buffer (100mM Tris pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 1% (v/v) Tween-20) for 10 min before being incubated at RT with NBT/BCIP (Roche) at 5µl/ml in alkaline buffer supplemented with levimasole (Vector, 1drop/5ml) to reduce background from endogenous alkaline phosphates. Sections were checked every hour until there was adequate staining. Sections were finally washed in T-PBS and fixed with PFA for 10 minutes, followed by 2 further PBS washes and mounted with a glycerol-based mounting medium (PBS:glycerol 1:4).

2.11 Human tissue collection

Adrenocortical tissues collected between 2008-2010 at St. Bartholomew's Hospital. Patients were consented prior to surgery and samples were collected from theatre and immediately preserved in RNAlater and placed in dry ice. Samples were then split, with some frozen in RNAlater and some fixed with formaldehyde and embedded in paraffin as described in Section 2.9.2

2.12 Data Analysis

All data were analysed using Graphpad Prism 7 and 8 to run student t-tests for direct comparisons. For group analysis ANOVA was used. Graphs were produced using Prism 8.

3 The presence of BMP3b and BMP2 in H295R cells and their effects on steroidogenesis

3.1 Aim

The aim of the work described in this chapter was to test the hypothesis that BMP3b and BMP2 are expressed in the H295R cells and hence in the adrenal cortex. Confirming these cytokines are present in H295R cells will provide a rationale to investigate their role in adrenal steroidogenesis. This will include analysis of potential interplay between BMP3b and BMP2 in controlling steroidogenesis and hence zonation of the adrenal cortex.

This chapter will concentrate on the effects of BMP3b and BMP2 overexpression and knockdown on expression of zone specific markers of the adrenal cortex and steroidogenesis in H295R cells.

3.2 Differentiation

Rat microarray data from the King laboratory indicated that *Bmp3b* was specifically expressed in the capsular/subcapsular region of the adrenal gland (zG/capsule fraction) and *Bmp2* was highly expressed in the inner cortex (which included zF, zR and medulla). There was a 12 fold increase in Bmp3b in the zG/capsular fraction compared to the inner cortex ($p < 0.01$) and a 2 fold increase in Bmp2 in the inner cortex compared to the zG/capsular fraction ($p<0.01$). The H295R cell line was used as a model to investigate the effect of cell differentiation in zG, zF or zR phenotypes when BMP3b or BMP2 protein was added. H295R cells were chosen for these experiments as they can express all the components of the steroidogenesis pathway (Gazdar et al., 1990; Rainey et al., 2004) as compared to other human adrenocortical cell lines available, which is discussed below.

The cell lines listed below were considered for the experiments:

3.2.1 SW13

SW13 is a cell line generated from a small cell adrenocortical carcinoma of a 55 year old female. These cells do not secrete any steroid products and were thus not suitable for this study (Leibovitz et al., 1973).

3.2.2 ACT-1

ACT-1 was a cell line derived from a 62 year old male with a large necrotic and haemorrhagic adrenocortical carcinoma and metastases. This cell line had limited use in this study to assess steroidogenesis due to the lack of CYP17 enzymes, which are necessary for the production of cortisol and androgens (Ueno et al., 2001).

3.2.3 RL-251

RL-251 is a human adrenal carcinoma cell line derived from a 75 year old male with cells that demonstrated abnormal steroidogenesis and was not responsive to ACTH stimulation, which again makes this cell line unsuitable for this study (Schteingart et al., 2001).

3.2.4 Immortalised inactivating PRKAR1A mutation cell line

The PRKAR1A inactivated adrenocortical cell line was derived from primary culture of a patient with primary pigmented nodular adrenocortical disease (PPNAD) and Carney's complex but this cell line was not used as it has been reported to lose its cortisol production soon after repeated passages (Nesterova et al., 2008).

3.2.5 Human adrenocortical carcinoma cell line (HAC)

The only other cell line that could have been considered in our study would have been the human adrenocortical carcinoma (HAC) cell line, in particular HAC15 which responded to Ang II, K^+ and ACTH (Parmar et al., 2008). It is a clonal cell line isolated from H295R and is more responsive to ACTH than H295R. However, at the time of this study, this cell line was not commercially available.

3.2.6 Other cell line attempts

Two other cell lines that do have the capacity for steroidogenesis are the paediatric adrenocortical adenoma cell line from a 1 year old female (Almeida et al., 2008) and Simian virus 40 (SV40) transformed cell line that produced human fetal adrenal cell clones (Hornsby et al., 1989). However, both of these cell lines have not been able to continue their steroidgenic capacity with ongoing passages, hence limiting their use.

3.2.7 H295R cell line

The human adrenal carcinoma cell line, NCI-H295R was used throughout this project. NCI-H295R was derived from the original cell line NCI-H295. NCI-H295 was first isolated and described by Gazdar *et al.,* in 1990. The cell line is derived from a 48 year old lady from the Bahamas who presented with clinical and biochemical features of adrenocortical carcinoma that later metastasised to the lungs and liver (Gazdar et al., 1990). However, this tumour was unusual in that it secreted a combination of glucocorticoid, mineralocorticoid and 17-ketosteroid. The cells expressed all the enzymes required for steroidogenesis (Staels et al., 1993). They were capable of producing their own cholesterol for steroidogenesis and the cells were inducible for steroid production with Ang II, potassium, fsk and to a lesser extent ACTH to shift the steroidogenic pathway in the direction to upregulate the steroid, dependent on the stimuli. Adding Ang II to H295 activates AT1 receptors resulting in an increase in intracellular calcium and up-regulation of *CYP11B2*, causing an increase in aldosterone production (Bird et al., 1993). Potassium increases aldosterone production by increasing intracellular calcium (Pezzi et al., 1997). Most likely due to the low expression of MC2R, ACTH appears to have a weak effect on H295 cells (Mountjoy et al., 1994). However, fsk, a cAMP activator, induces *CYP11B1* and *CYP17* transcription. This results in increased cortisol production initially (Denner et al., 1996) but chronic Fsk stimulation leads to activation of the androgen pathway with the production of DHEA (Cobb et al., 1996).

Although this original H295 cell line was able to produce all the products of steroidogenesis, the cell line itself had a slow doubling time of 5 days (Rainey et al., 2004). Therefore, a strain, known as H295R cells were selected for a shorter doubling time of 2 days by the addition of Ultroser G in the media (Hornsby and McAllister, 1991; Rainey et al., 2004). H295R was also

able to grow as an adherent monolayer, unlike H295 which only grew as loosely attached cell clusters. H295R is able to express all the enzymes required for steroidogenesis, responds appropriately to Ang II, K^+ and Fsk, representing the three zones of the adrenal cortex and is the most studied adrenocortical cell line to date. Another strain of H295 is H295A, which again is able to produce all the steroids but it is not responsive to Ang II.

3.2.8 Zone specific expression of BMP3b

The following experiments investigated the effect of BMP3b on the expression of key steroidogenic genes (*CYP11B1, CYP11B2, CYTB5, SULT2A1*) in the three zones of the adrenal cortex (zG, zF and zR).

The effects of adding BMP3b to H295R cells, over-expressing BMP3b and then silencing to see if the effects can be reversed was investigated. cDNA was made following treatment of cells as described in Section 2.4. RT-qPCR was performed to compare the relative mRNA levels of enzymes of steroidogenesis, with or without treatment.

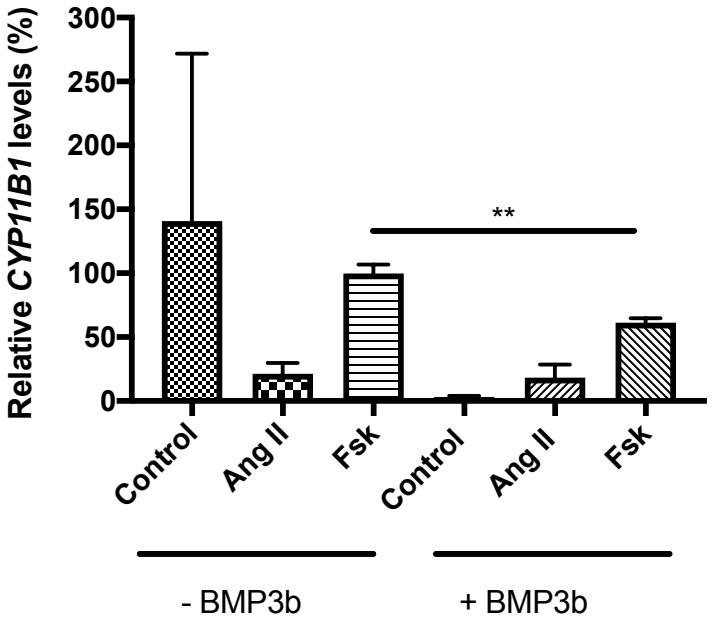


Figure 3.1 *CYP11B1* expression in H295R cells treated with BMP3b
H295R cells were treated with either recombinant BMP3b or a vehicle only control. *CYP11B1* expression in H295R cells with or without BMP3b treatment were compared with control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM. **p<0.01, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

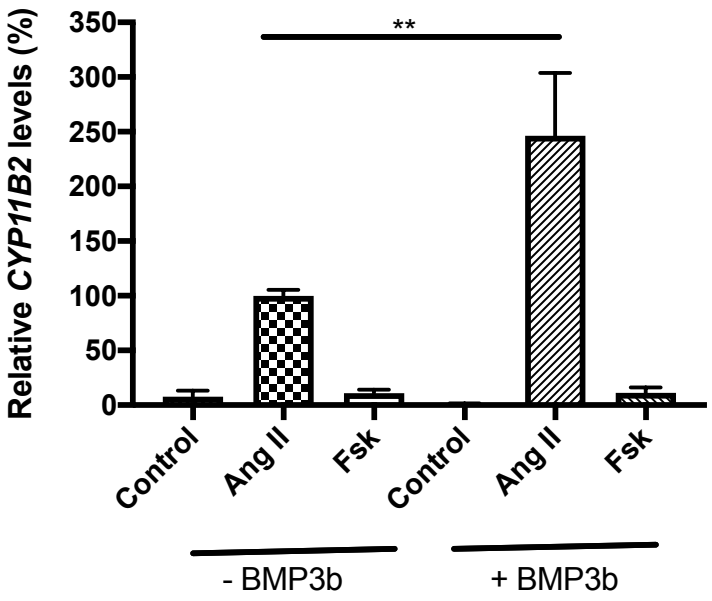


Figure 3.2 *CYP11B2* expression in H295R cells treated with BMP3b
H295R cells were treated with either recombinant BMP3b or a vehicle only control. *CYP11B2* expression in H295R cells with or without BMP3b treatment were compared with control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM. **p<0.01, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

Figure 3.1 and Figure 3.2 have demonstrated a significant effect on expression of markers of steroidogenesis with the addition of BMP3b protein to H295R cells. BMP3b causes significantly increased mRNA levels of *CYP11B2* ($p<0.01$) in cells treated with Ang II and significantly reduced *CYP11B1* mRNA levels ($p<0.01$) in cells treated with Fsk. The use of recombinant protein was informative but further investigation was necessary to confirm this effect. This was achieved by experiments overexpressing *BMP3b.* H295R cells were transfected with either an empty vector (pIRES) as control or a vector for expression of full length human *BMP3b* cDNA.

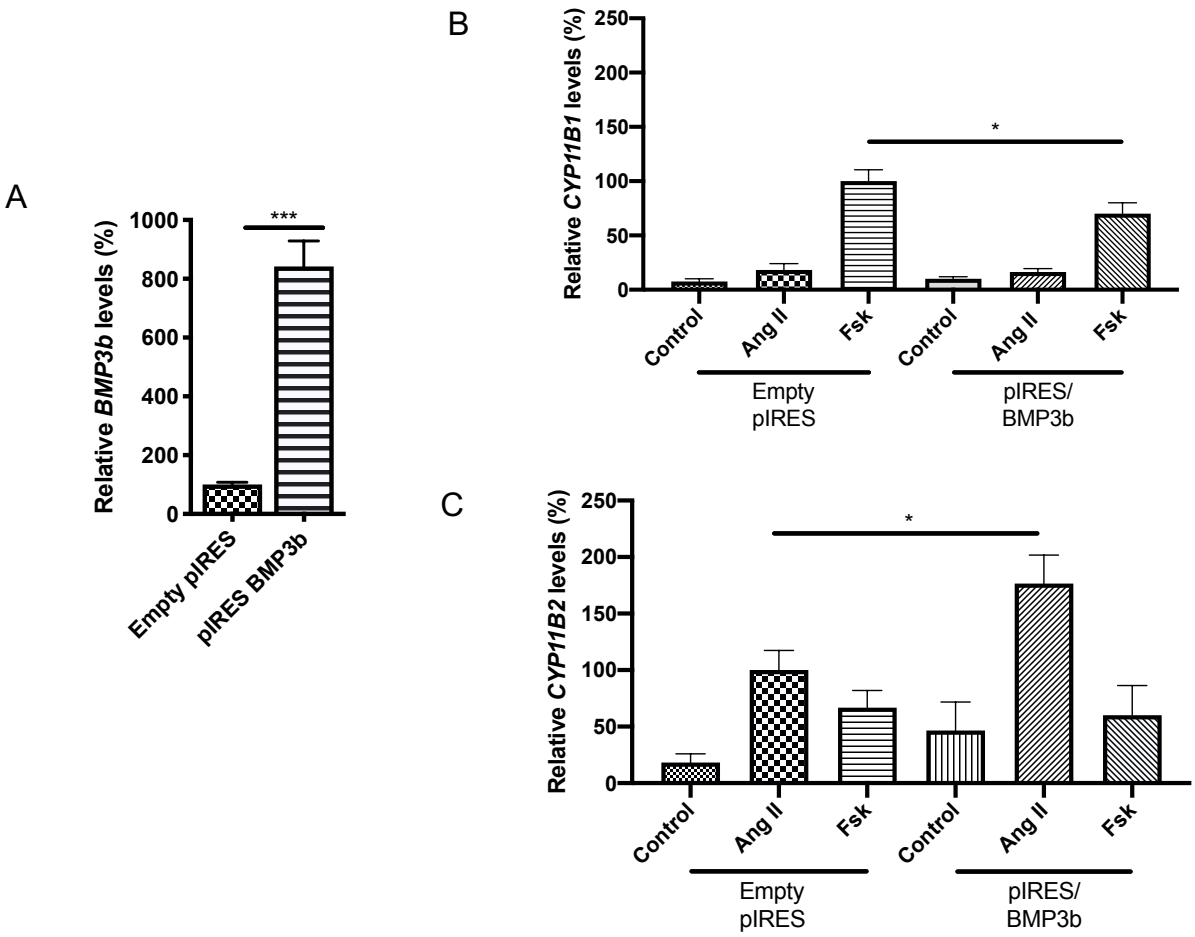


Figure 3.3 *BMP3b, CYP11B1* and *CYP11B2* expression in H295R cells stably expressing BMP3b

H295R stably expressing BMP3b cells were generated by transfection with a pIRES vector containing a full length human BMP3b cDNA and subsequent antibiotic selection. A control line transfected with empty pIRES vector was made at the same time (A) *BMP3b* is significantly over expressed in stable cells compared with control cells, as indicated by elevated levels of BMP3b RT-pCR product. (B)(C) *CYP11B1* and *CYP11B2* expression in BMP3b over expressing H295R cells compared with control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, ***$p<0.001$, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

The results shown in Figure 3.3 are consistent with published data on the effects of Ang II and fsk on H295R cells (Liakos et al., 2003). Furthermore, H295R cells treated with Ang II have significantly increased levels of *CYP11B2* mRNA expression ($p<0.05$) and decreased levels of *CYP11B1* mRNA ($p<0.05$), when treated with Fsk, when BMP3b is overexpressed.

Zonation theories, as discussed in section 1.3, suggests that cells migrate in a centripetal fashion from the capsule towards the medulla. As the cells travel inwards, their steroidogenic identity changes. Many genes play a role in this process and have been discussed previously. With the prevalence of BMP3b in the zG and not in the inner cortex, we set out to investigate whether the presence or absence of BMP3b would have an effect on the steroidogenic expression of H295R cells into a zR phenotype. Cytochrome b5 (*CYTB5*) and sulfotransferase family 2A member 1 (*SULT2A1)* are both important for the production of androgens (DHEA and DHEAS). Treatment of H295R cells with recombinant BMP3b resulted in a significant decrease in levels of *CYTB5* ($p<0.01$) and *SULT2A1* ($p<0.05$) mRNA (Figure 3.4). This suggests BMP3b may have a suppressive effect on zR specific genes.

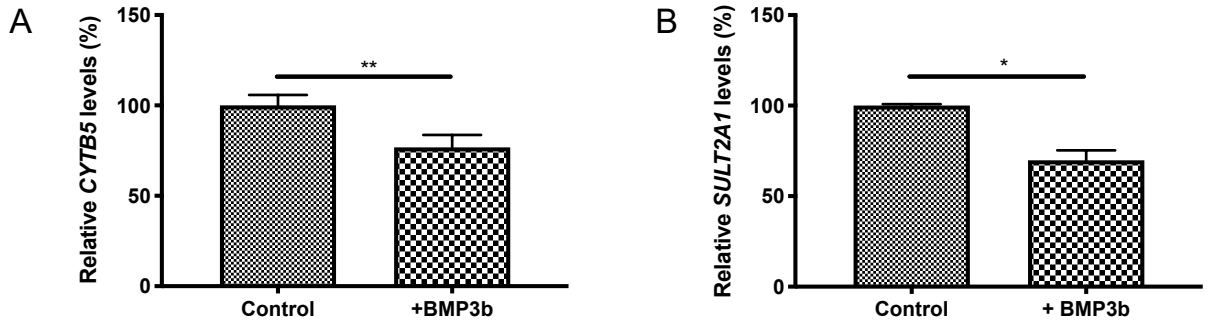


Figure 3.4 *CYTB5* and *SULT2A1* expression in H295R cells treated with BMP3b
H295R cells were treated with either recombinant BMP3b or a vehicle only control. (A)(B) *CYTB5* and *SULT2A1* expression in H295R cells with or without BMP3b treatment were compared with control cells. N=3, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, **$p<0.01$, student's T-Test.

Experiments using overexpression and recombinant BMP3b all showed that BMP3b protein can significantly increase levels of *CYP11B2* and decrease levels of *CYP11B1* mRNA expression in H295R cells undergoing different differentiation paradigms. Treatment with BMP3b protein also decreased expression of the zR specific genes *CYTB5 ($p<0.01$)* and *SULT2A1* ($p<0.05$). This significant downregulation of *CYTB5* and *SULT2A1* mRNA

expression could indicate that BMP3b, which was not suggested to be expressed in the inner zones of the adrenal cortex, based on our microarray data, can exert an effect on zR specific genes.

To further explore the importance of BMP3b for the up and down regulation of steroidogenesis genes, a knockdown study was designed using siRNAs targeting BMP3b. These knockdown experiments were performed with addition of Ang II or Fsk at the time of transfection,

The knockdown experiments shown in Figure 3.5 and Figure 3.6 show that reducing RNA levels of *BMP3b* resulted in significant upregulation of *CYP11B1* ($p<0.05$) and *CYTB5* *($p<0.05$)*. These results suggest that BMP3b expression may prevent mRNA expression of *CYP11B1* and *CYTB5*, and is thus acting as a negative regulator of differentiation to a zF and zR phenotype. However, there was no effect shown of siBMP3b on *SULT2A1* mRNA expression. This may be because the magnitude of the knockdown (approximately 60% reduction in mRNA) was insufficient to alleviate any negative regulation of expression in this case. There was also no significant downregulation of *CYP11B2* seen here, which does not support the hypothesis that BMP3b could play a key role in promoting the zG phenotype within the adrenal cortex but may also be a consequence of the incomplete knockdown.

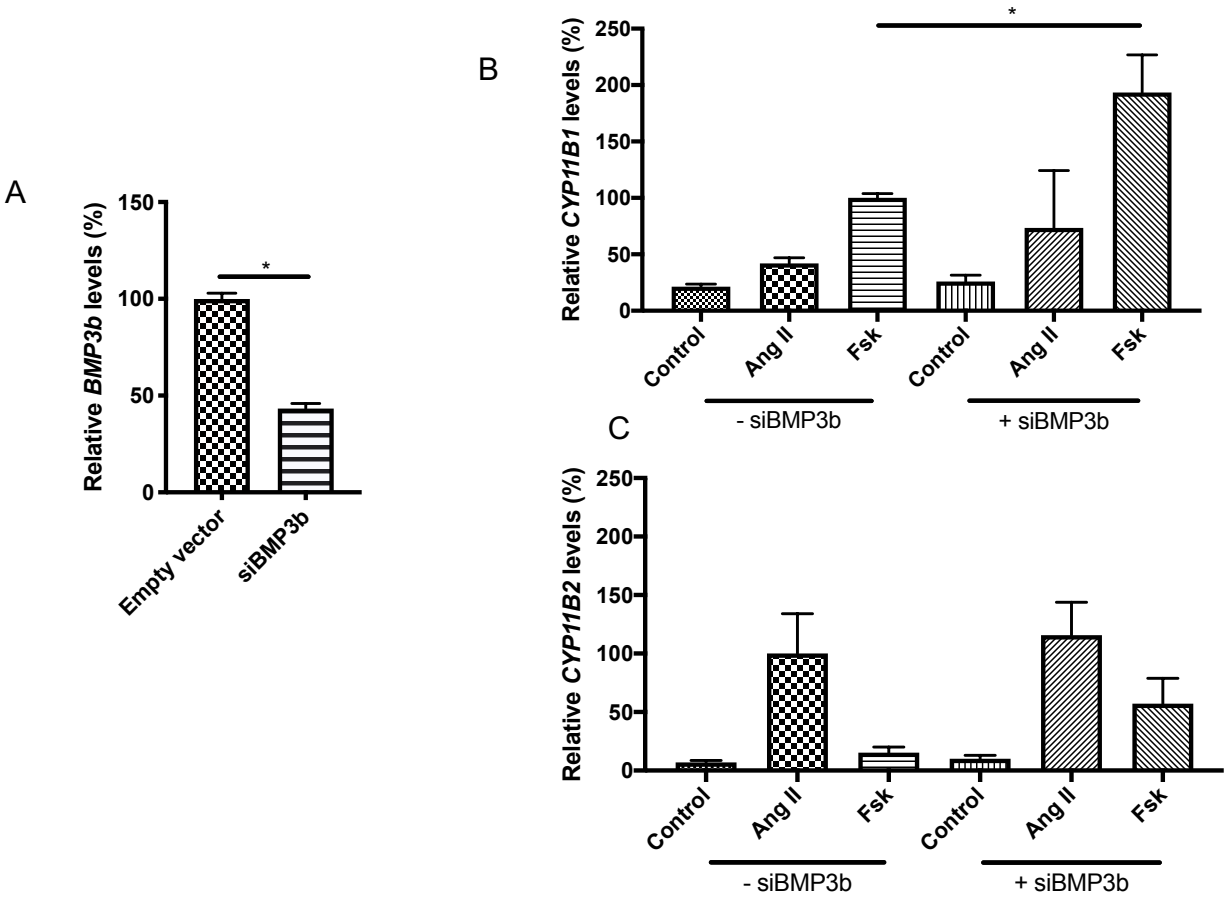


Figure 3.5 *BMP3b, CYP11B1* and *CYP11B2* expression in H295R cells transfected with siBMP3b

H295R cells were transfected with either a non-targeting control siRNA or an siRNA targeting BMP3b. (A) *BMP3b* expression was significantly reduced in siBMP3b transfected H295R cells compared cells transfected with the control siRNA. (B)(C) *CYP11B1* and *CYP11B2* expression in siBMP3b transfected H295R cells were compared with controls in cells treated with Ang II or Fsk. N=6, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

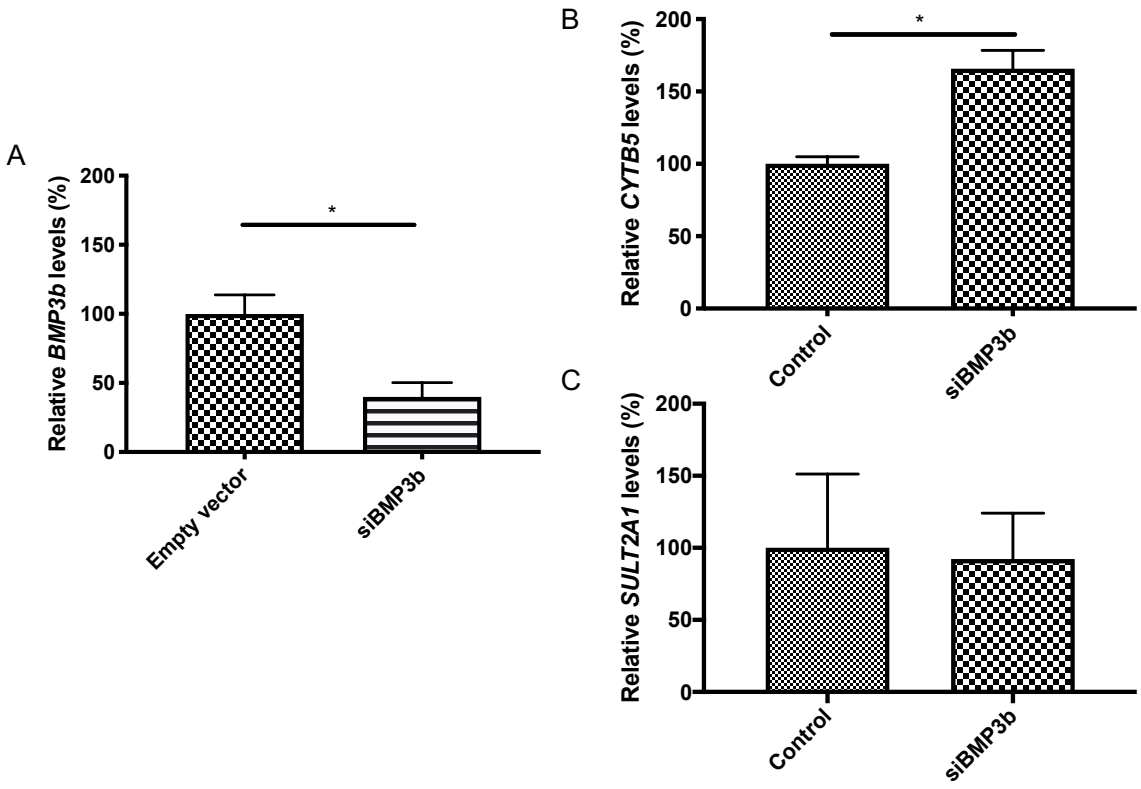


Figure 3.6 *CYTB5* and *SULT2A1* expression in H295R cells transfected with siBMP3b
H295R cells were transfected with either a non-targeting control siRNA or an siRNA targeting BMP3b (A). *BMP3b* expression was significantly reduced in siBMP3b transfected H295R cells compared to controls. (B)(C) *CYTB5* and *SULT2A1* expression in siBMP3b transfected H295R cells compared with empty vector control and in response to Ang II or Fsk. N=6, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

The data so far have shown that BMP3b, by adding recombinant protein or through overexpression, significantly increases angiotensin-induced *CYP11B2* mRNA expression ($p<0.01$ and $p<0.05$, respectively), indicating that it could have a direct effect on differentiation of H295R cells to the zG phenotype. Conversely, BMP3b had an inhibitory effect on *CYP11B1* mRNA expression, which is found within the zF.

In conclusion, this set of experiments have shown that BMP3b affects steroidogenic gene expression in H295R cells. Taken together, the results are consistent with the ability of BMP3b to upregulate *CYP11B2* expression, a marker of the zG phenotype, and downregulate the zF and ZR phenotype as assessed by *CYP11B1* and *CYTB5* expression, respectively. It is tempting

to speculate that BMP3b can promote the zG phenotype and/or inhibit the conversion of the cells to the zF/zR phenotype.

3.2.9 Zone specific expression of BMP2

Microarray studies have indicated that *BMP2* is expressed at higher levels in the inner zones of the rat adrenal compared to outer zones. H295R cells were treated with BMP2 protein to investigate the effects on steroidogenic gene expression of all 3 cortical zones and compare them to the effects of opposite BMP3b.

Figure 3.7demonstrated that BMP2 treatment of H295R cells did not have any significant effect on *CYP11B1* or *CYP11B2* mRNA expression, indicating that it may not have a direct or indirect role in differentiation towards or steroidogenesis in the zF or zG respectively. Moreover, microarray data indicated that *Bmp2* was most highly expressed in the inner cortex of the rat adrenal, which include zF, zR and the medulla. Therefore, zR specific enzymes were investigated with BMP2 treated cells (Figure 3.8). The results showed that both *CYTB5* and *SULT2A1* mRNA expression were significantly upregulated in the presence of BMP2 ($p<0.05$ for both) compared with control.

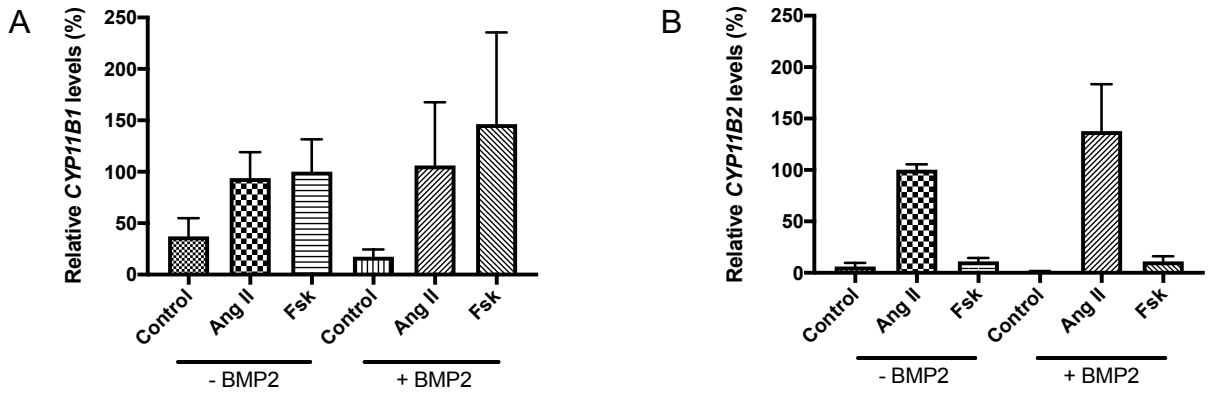


Figure 3.7 CYP11B1 and CYP11B2 expression in BMP2 treated H295R cells
H295R cells were treated with either recombinant BMP2 or a vehicle only control. (A) (B) *CYP11B1* and *CYP11B2* expression in H295R cells with or without BMP2 treatment were compared with control cells and in response to Ang II or Fsk. N=6, performed in triplicates. Error bars indicate SEM. Student's T-Test used. Ang II = angiotensin II, Fsk = forskolin

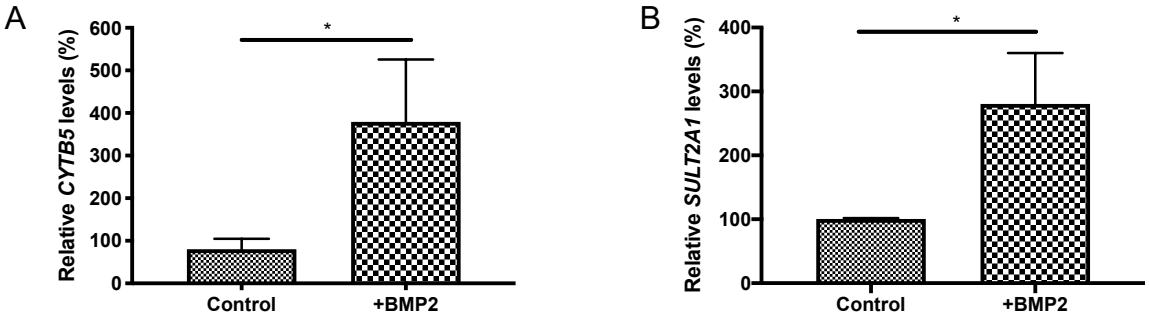


Figure 3.8 CYTB5 and SULT2A1 expression in BMP2 treated H295R cells
H295R cells were treated with either recombinant BMP2 or a vehicle only control. (A)(B) *CYTB5* and *SULT2A1* expression in H295R cells with or without BMP2 treatment were compared with control cells. N=6, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, student's T-Test.

In conclusion, these experiments showed that BMP2 did not exert any significant effect on *CYP11B1* or *CYP11B2* mRNA expression. However, it did show a significant upregulation of zR specific enzymes ($p<0.05$),. This finding suggests that BMP2 may have a local role in promoting differentiation into a zR phenotype in normal adrenal zonation and differentiation. Limitations to these experiments are discussed in the general discussion.

3.3 Steroid hormone production

These experiments indicate a role of BMP3b in promoting a zG phenotype, while BMP2 promotes a zR phenotype respectively. It indicates that BMP3b and BMP2 can both have an effect on androgen steroidogenesis. BMP3b could be a factor that negatively affects differentiation into a zR phenotype, which could be through suppression of BMP2 expression or action, as shown in previous experiments. However, from these results, it was unclear if the interplay between BMP3b and BMP2 results in significant steroid production in H295R cells.

Changes in mRNA expression do not necessarily result in alterations in protein expression of a similar magnitude. To investigate the correlation of mRNA expression with BMP protein levels, ELISA (Enzyme-linked immunosorbent assay) was performed.

From the previous experiments, it was predicted that aldosterone would be highest in the Ang II +/- BMP3b treated group and would be unaltered with BMP2 presence. The results shown below in Figure 3.9 did not show the predicted pattern. Aldosterone production was not significantly increased or decreased in any of the groups. Thus the ELISA data did not correlate with our qPCR results. Cortisol and DHEAS was also evaluated and significant differences were not found (Figure 3.10 and Figure 3.11).

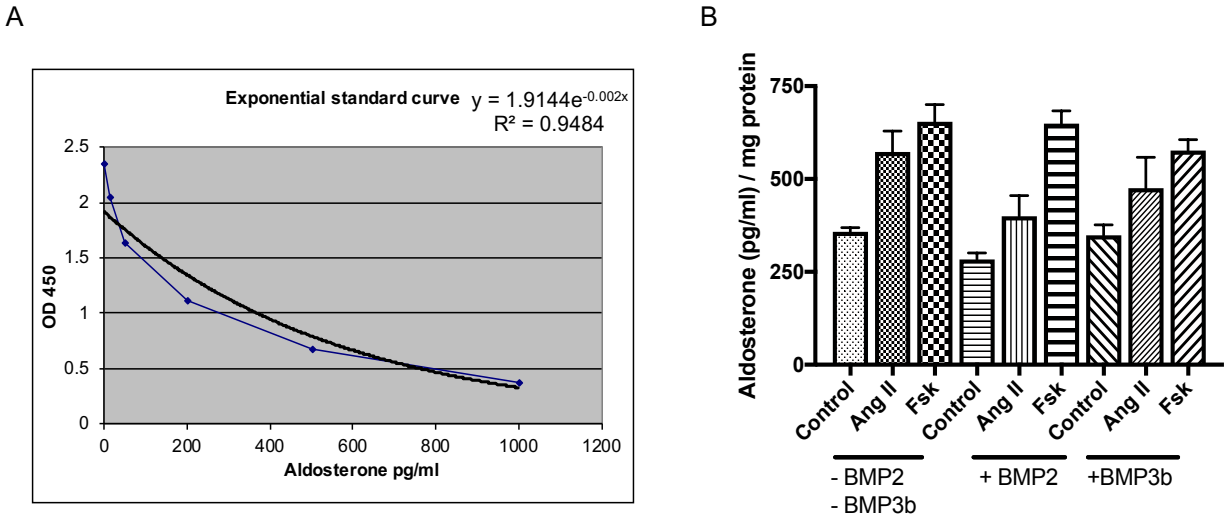


Figure 3.9 Aldosterone production in H295R cells treated with BMP2 or BMP3b
H295R cells were treated with either recombinant BMP2, BMP3b or a vehicle only control. (A) Standard curve was generated to calculate the production of aldosterone. (B) Aldosterone levels were measured H295R cells treated with BMP2, BMP3b compared to control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM. Student's T-Test used. Ang II = angiotensin II, Fsk = forskolin

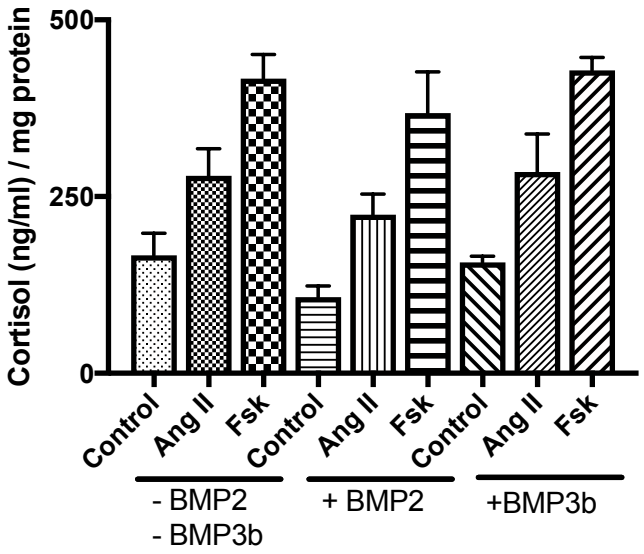


Figure 3.10 Cortisol production in H295R cells treated with BMP2 or BMP3b
H295R cells were treated with either recombinant BMP2, BMP3b or a vehicle only control. Cortisol levels were measured in H295R cells treated with BMP2, BMP3b compared to control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM. Student's T-Test used. Ang II = angiotensin II, Fsk = forskolin

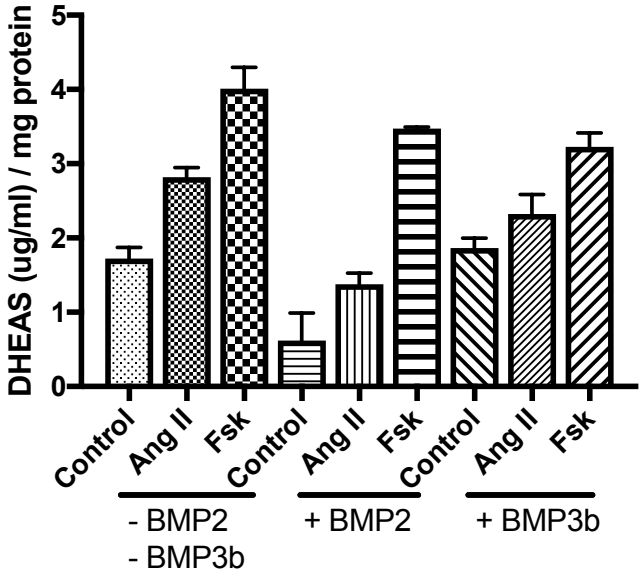


Figure 3.11 DHEAS production in H295R cells treated with BMP2 or BMP3b
H295R cells were treated with either recombinant BMP2, BMP3b or a vehicle only control. DHEAS levels were measured in H295R cells treated with BMP2, BMP3b compared to control cells and in response to Ang II or Fsk. N=3, performed in triplicates. Error bars indicate SEM. Student's T-Test used. Ang II = angiotensin II, Fsk = forskolin

The results of steroid hormone production from the ELISA experiments were not consistent with the RT-qPCR findings with BMP3b and BMP2. No clear pattern was seen in hormone

production that would indicate that BMP3b was positively affecting aldosterone production or negatively affecting cortisol or DHEAS production, or that BMP2 was positively affecting DHEAS production. There were limitations to this direct comparison, as BMPs are not the only pathway in adrenal steroid production. Other mechanisms and pathways are likely to be involved to maintain appropriate steroid levels and homeostasis, which have not been accounted for in this study design. Therefore, mRNA expression shown previously cannot be directly corrrelated to increased protein production or function.

The effect of BMP3b and BMP2 on steroid production was investigated with knockdown experiments using siBMP3b and siBMP2 (Figure 3.12, Figure 3.13 and Figure 3.14).

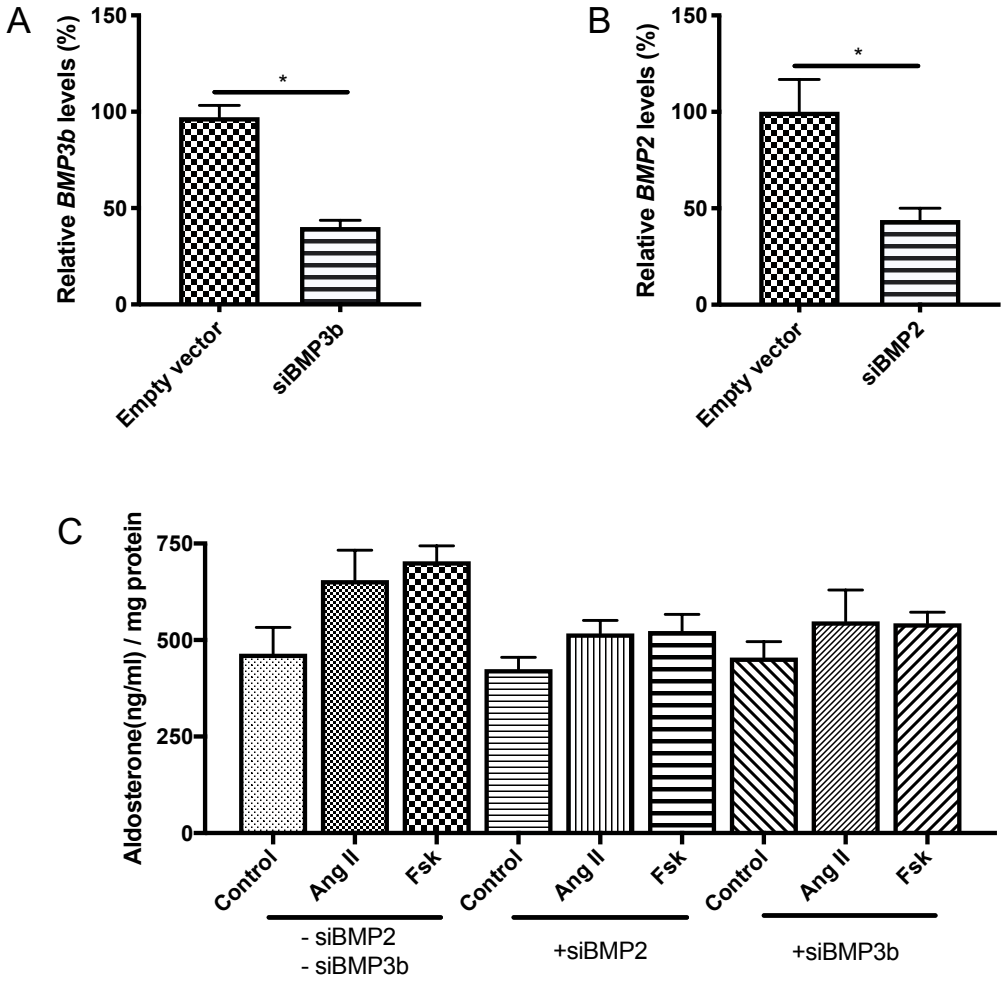


Figure 3.12 Aldosterone production in H295R cells transfected with siBMP3b or siBMP2

H295R cells were transfected with either a non-targeting control siRNA or siRNAs targeting BMP3b or BMP2. (A)(B) *BMP3b* and *BMP2* expression was significantly reduced in siBMP3b and siBMP2 transfected H295R cells, compared to controls. (C) Aldosterone production was measured in siBMP3b and siBMP2 transfected H295R cells compared with non-targeting control siRNAs and in response to Ang II or Fsk.

N=3, performed in duplicates. Error bars indicate SEM.

*$p<0.05$, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

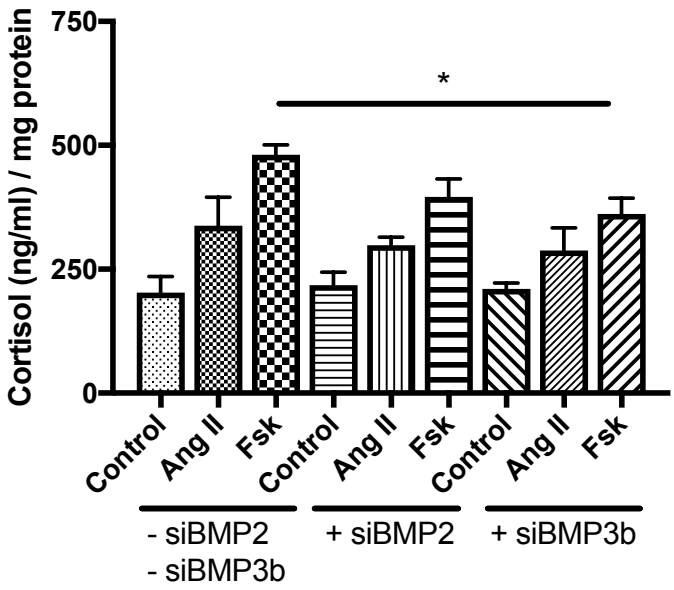


Figure 3.13 Cortisol production in H295R cells treated with siBMP2 or siBMP3b
H295R cells were transfected with either a non-targeting control siRNA or siRNAs targeting BMP3b or BMP2. Cortisol production was measured in siBMP3b and siBMP2 transfected H295R cells compared with non-targeting control siRNAs and in response to Ang II or Fsk. N=3, performed in duplicates. Error bars indicate SEM.
*p<0.05, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

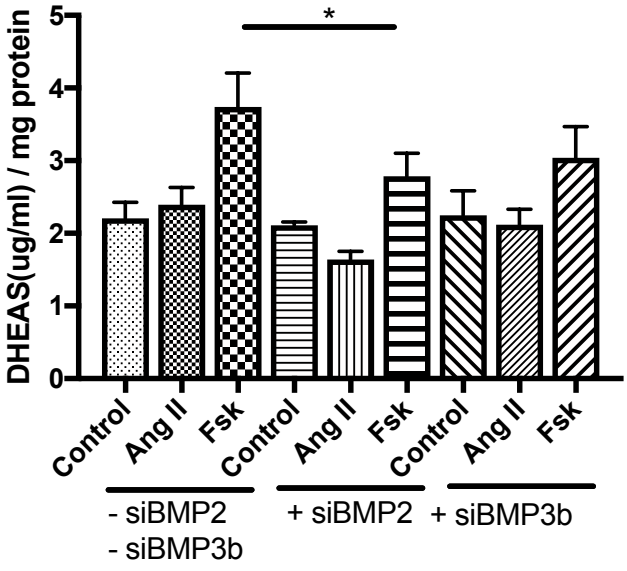


Figure 3.14 DHEAS production in H295R cells treated with siBMP2 or siBMP3b
H295R cells were transfected with either a non-targeting control siRNA or siRNAs targeting BMP3b or BMP2. DHEAS production was measured in siBMP3b and siBMP2 transfected H295R cells compared with non-targeting control siRNAs and in response to Ang II or Fsk. N=3, performed in duplicates. Error bars indicate SEM.
*p<0.05, student's T-Test, Ang II = angiotensin II, Fsk = forskolin

There was a significant difference in the production of cortisol and DHEAS with the presence of siBMP3b and siBMP2 ($p<0.05$ for both), repectively. The latter result supports a role for BMP2 in zR phenotype establishment and steroidogenesis, but the former is a surprising result which is at odds on the previous data indicating that BMP3b should be inhibitory to the establishment of the zF phenotype.

3.4 Discussion

These results correlate with the rat microarray data (from the King group), which found BMP3b exclusively in the outer cortex in the region of the stem/progenitor cells. This suggests BMP3B is likely to exert its effects locally. However, in the H295R cell model it can also suppress markers of the zR: *CYTB5* and *SULT2A1.* As a secreted molecule, it is possible that BMP3b could have an effect on the outer zF, but is unlikely to affect the zR in any appreciable manner, other than inhibiting the differentiation of progenitor cells to the zF/zR commitment pathway. The suppressive effects were significantly reversed for *CYP11B1 ($p<0.05$)* and *CYTB5* ($p<0.05$) when BMP3b was knocked down with siBMP3b.

H295R cells express components of the BMP pathway and these have an effect on steroidogenesis. Previous data, suggesting that expression of BMP3b occurs exclusively in the outer zone of the adrenal, while BMP2 is expressed highly in the inner zone, has led to the hypothesis that within the cortex BMPs exert specific effects within zonal area that may contribute to the process of zonation. Indeed, BMPs have been shown to have a zonal effect in other studies (Liakos et al., 2003; Suzuki et al., 2004) and hence it is likely that, like BMP6, BMP3b acts within the zG (Inagaki et al., 2006). BMPs are morphogens, thus the finding of zone specific BMPs fits in with the hypothesis of BMP3b being specific to zG and BMP2 to zF/zR. However, their effects can be observed beyond the zones as they can work on a gradient dependent mechanism (Tabata and Takei, 2004). Adrenocortical cell lines were used to allow for the investigation of steroidogenic gene expression on a mRNA level. Due to the lack of suitable antibodies available at the time of the experiments, it was not possible to investigate

protein expression using Western blotting techniques. Ideally these experiments would be repeated to measure protein if suitable antibody tools become available.

These experiments have shown that *BMP3b* and *BMP2* are likely to have distinct regions of expression within the adrenal cortex and that BMP3b treatment of H295R cells can inhibit their conversion to the zF/zR phenotype. These data are consistent with a hypothesis that BMP3b is inhibitory to cell differentiation by inhibiting the expression and action of BMP2 (Figure 1.6 and Figure 1.7). It is hypothesised that as cells migrate from the capsule into the cortex they are exposed to increasing BMP2 signals which promote the differentiation of the cells to the zF and zR phenotypes. A possible model of BMP signalling in the adrenal cortex is the establishment of a BMP3b gradient exclusively in the outer cortex. This gradient antagonises BMP2 function, thus maintaining the phenotype of precursor cells. As these cells proliferate and migrate centripetally, they exit the zG and hence BMP3b gradient. This results in BMP2 exposure with a change in phenotype to zF or zR (Figure 3.15).

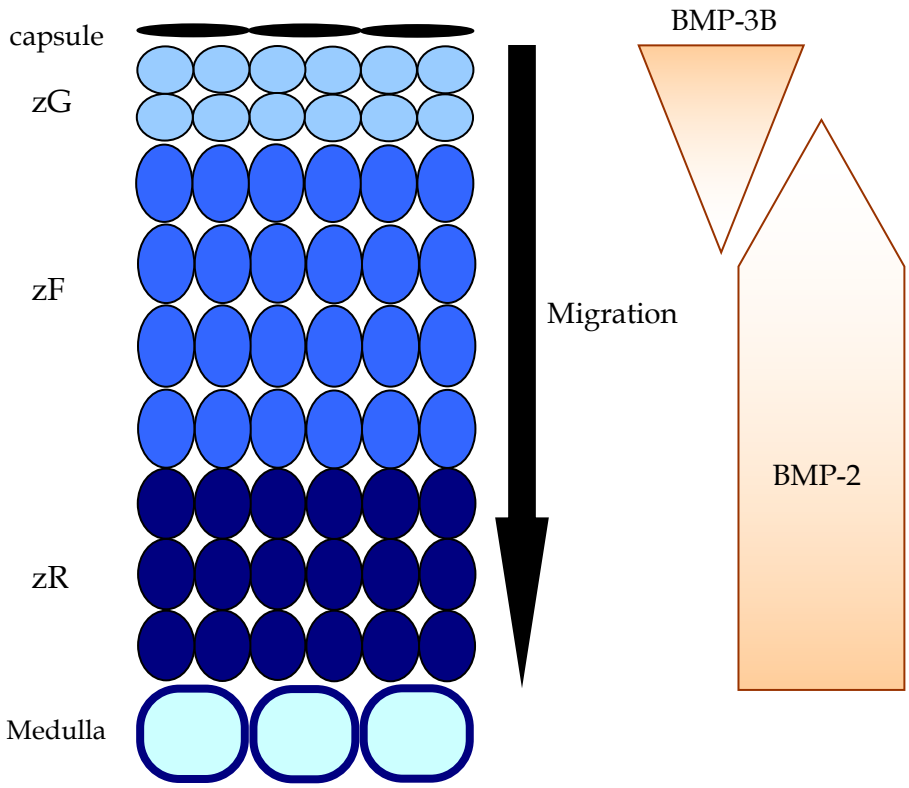


Figure 3.15 Model for the structural and functional zonation in the adrenal gland
A BMP3b gradient is established in the outer cortex. This gradient antagonises BMP2 expression thus maintaining the phenotype of the precursor cells in the outer zones of the adrenal cortex. These cells proliferate and migrate into the inner cortex. As they leave the zG, they exit the BMP3b gradient and become subject to the actions of BMP2 or other factors. The cells differentiate into other cortical phenotypes and eventually die at the ZR/medulla boundary.

This proposed model was based on mRNA levels detected in H295R cells. Experiments in H295R cell using ELISA steroid assays demonstrated that the addition of Fsk increased cortisol production ($p<0.05$) and the addition of siBMP2 increased DHEAS production ($p<0.05$) which support the qPCR findings of mRNA levels. However, the effects of BMP3b and BMP2 on aldosterone and DHEAS production respectively were not significant and so did not reflect the qPCR results to fully support this model on a functional basis. Interestingly, the presence of siBMP3b decreased cortisol production significantly ($p-<0.05$), which contradicts the qPCR results of increased levels. However, siBMP3b significantly increases *CYTB5* expression in these cells (Figure 3.6) and it is possible that reduced cortisol production reflects the

preferential androgen production. DHEAS was not detected in the H295R cells by ELISA. This could be explained by previous results where siBMP3b had no significant effect on *SULT2A1* expression, a key enzyme for the production of sulphated DHEA. Therefore, if sulphation did not occur, then it is plausible that DHEAS would not be detected. In addition, Ang II presence did not significantly increase aldosterone production. A possible explanation is that these experiments did not take into account other BMPs present in the cortex, such as BMP6, which may have also had an effect on the synthesis of aldosterone in all conditions. Another possibility could be the kit used, which is only valid for human plasma or serum and not specifically for culture medium, though ELISA measurements are used in many cell culture studies. A third reason could be a compensatory mechanism that could exist in H295R cells to maintain steroid production, which has been shown in bone formation and fracture repair, where the lack of BMP7 is compensated by BMP4 (Tsuji et al., 2010). More tests with different assay platforms and more samples should have been used to further validate the findings in these experiments.

Further experiments are needed to look at the interaction between *BMP3b* and *BMP2,* with the inclusion of all the other BMPs found in the adrenal cortex in order to demonstrate a direct effect.

BMP3b is expressed in the outer capsular/subcapsular region of the cortex and is able to inhibit the differentiation of H295R cells to the zF/zR phenotype, suggesting that it may be a marker of stem or progenitor cells or can influence their differentiation. H295R cells were a useful cell line in which to test this hypothesis as it is a relatively undifferentiated cell line with the capacity to differentiate into all cell types of steroidogenesis and is able to replicate infinitely. However, as H295R cells are derived from a carcinoma, further passaging of these cells can lead to new mutations and the cell characteristics may change over time, with differentiation and de-differentiation at an unknown rate. This was avoided in our experiments by limiting the passages of cells used. Ideally, to investigate stem cell progenitors, human primary adrenal cultures should be use. This was considered but thought not to be feasible because of technical and ethical issues. For this reason, H295R cells (or different undifferentitated cell lines that may be available in the future) remain the best model from which to gather initial data. Other

cell lines, such as HAC15, are also available (Parmar et al., 2008). HAC15, like H295R cell line, is derived from human adrenocortical carcinoma cells that is thought to be both ACTH and Ang II responsive, whereas H295R cells are less ACTH responsive. However, HAC has been shown to have been derived from monoclones of H295R cells, therefore this is not a totally novel cell line to corroborate the current data. One possible approach in the future, when primary adrenal cell cultures are more easily accessible, more studies can be carried out over a longer period of time with the use of hTERT (human telomerase reverse transcriptase). hTERT has the ability to extend the life span of normal human cells by overcoming the problem of telomere shortening without causing cellular transformation and genomic instability (as would be expected in cancer cell lines) (Ouellette et al., 2000).

Many studies on BMPs have demonstrated the vast number of roles and mechanisms of how BMPs can exert their effects in a local and gradient fashion, such as BMP4 in gastrulation (Jones and Smith, 1998). Nilsson described the differences in concentration of various BMPs in the different zones of growth plate, contributing to the maintenance of the cells in their stem cell, proliferative or hyperplastic state (Nilsson et al., 2007). This morphogen action also applies to drosophila where the BMP homolog, decapentaplegic (DPP) interacts with hedgehog (HH), to direct anteroposterior patterning of the wing with differing expression concentrations (Tanimoto et al., 2000). The role of BMPs can be very tissue and cell specific, making extrapolation of findings in one organ to another difficult (Wang et al., 2014). In conclusion, the *in vitro* studies have demonstrated that *BMP3b*, which was originally found in the capsular region of the rat microarray studies, act on H295R cells to increase *CYP11B2* mRNA expression. It also has exerts the opposite effect to BMP2, which is found in the inner cortex of the rat adrenal, on *CYTB5* and *SULT2A1* (zR specific) mRNA expression.

The adrenal gland is such a complex and vital organ for survival, that it is highly unlikely for only one mechanism to have evolved for its zonation and maintenance in function. Published data suggests that Rspo3, Wnt, Shh and PKA pathways are all required for the regeneration and zonation of the adult adrenal cortex (Drelon et al., 2016a; Vidal et al., 2016). The data also support a centripetal model of recruitment of progenitors cells in the capsular region that are then differentiated to a zG phenotype initially then to zF phenotype by lineage conversion

(Vidal et al., 2016). The published data suggests that Rspo3, found in the adrenal capsule induces β-catenin signalling which then activates Shh signalling. Shh signalling is essential for recruitment of capsular cells (King et al., 2009), as a lack of Shh signalling will result in impaired adrenal cortical growth. Shh activation will then activate Gli1 signalling in the capsular region to initiate cell recruitment for differentiation (Vidal et al., 2016). Following cell recruitment, the PKA pathway appears to be important for lineage conversion of zG cells to the zF phenotype. Activation of PKA also prevents zG differentiation through Wnt4 repression and inhibition of the Wnt pathway (Drelon et al., 2016a). There is also interplay between PKA and MRAP, an accessory protein that triggers the cAMP/PKA pathway and the recent study by Novoselova *et al*., demonstrated that a MRAP knock out mouse has impaired adrenal progenitor cell differentiation and gland zonation (Novoselova et al., 2018). These pathways all have a role to play in adrenal renewal and zonation but this is unlikely to be the only pathways involved, as more association between pathways are being discovered. BMPs are known to have interplay with the Hh and Wnt pathway in other organs (Ding and Wang, 2017; Hu et al., 2005; Sanz-Ezquerro et al., 2017) and it could be postulated that BMPs may also have a role in adrenal zonation through interaction with any of these pathways, which would need further studies.

Although my data support that BMP3b and BMP2 could be pathways affecting adrenal zonation and steroidogenesis, further experiments and controls would be required for definitive proof. These would include:

1. To normalise qPCR to additional reference genes because this increases the accuracy of data with geometric averaging. Vandesompele *et al.,* demonstrated that using single reference genes for normalisation can lead to erroneous normalisation of up to 3 and 6.4 fold in 25% and 10% of cases, respectively. Some reference genes were shown to be highly variable in different tissue types and could also be unstable (Vandesompele et al., 2002).
2. To demonstrate transfection efficiency with either co-transfection with Renilla DNA and a luciferase assay or by GFP immunofluorescence. This was not performed in my samples as the initial experiments for optimisation of transfection efficiency was

performed by my predecessor. Therefore they were not repeated for these experiments as the reagents and BMP plasmids used were identical. Future experiments should be with transfection efficiency reported in order to confirm my current findings. However, if transfection efficiency were to be carried out with each transfection, there is no methodology to correct for the different transfection rates in relation to the results obtained for each experimental replicate. My experiments were carried out with consistent methods and parameters to minimise variability between experiments.

3. Immunoblot to confirm the knockdown efficiency of siRNAs used to demonstrate more than 50% reduction in protein levels. siScrambled should have been used in all the knockdown experiments to show that the results were not a result of transfection methods. It should be noted that in similar studies by Dr Bakmanidis a scrambled control had no effect on steroidogenic gene expression in this cell line nor did a GAPDH targeted siRNA (data not shown). However, given that the results obtained with validated siRNAs were in almost all cases the opposite of those seen with overexpression of the BMP and/or treatment with recombinant protein, it would be highly unlikely that the siRNA results presented here were as a consequence of off target effects. Future experiments using all these parameters to demonstrate knockdown would be ideal.

4 Localisation of BMP3b and BMP2 in the adrenal cortex

4.1 Aim

Real-time PCR has established that BMP3b and BMP2 mRNAs are expressed in the outer and inner zones of the rat adrenal cortex respectively. This chapter will investigate the location of *Bmp3b* and *Bmp2* expression in rat and mice adrenal glands, using adrenal glands from Wistar rats *and Bmp3b* knock out mice. The localisation of BMP receptors will also be investigated to identify where Bmp3b and Bmp2 may exert their effects.

Due to the lack of good antibodies for detection of BMPs and their receptors in the adrenal cortex, a non radioactive *in situ* hybridisation (ISH) approach Figure 4.1 was used to look for endogenous mRNA expression. In this technique, the probe was labelled with digoxigenin, that will stain a magenta colour upon mRNA binding to the probe. Experiments were carried out with a 'sense' negative control, as shown in Figure 4.2 to ensure that any positive staining in tissues is specific to the probe, showing true expression.

4.2 *In situ* hybridisation

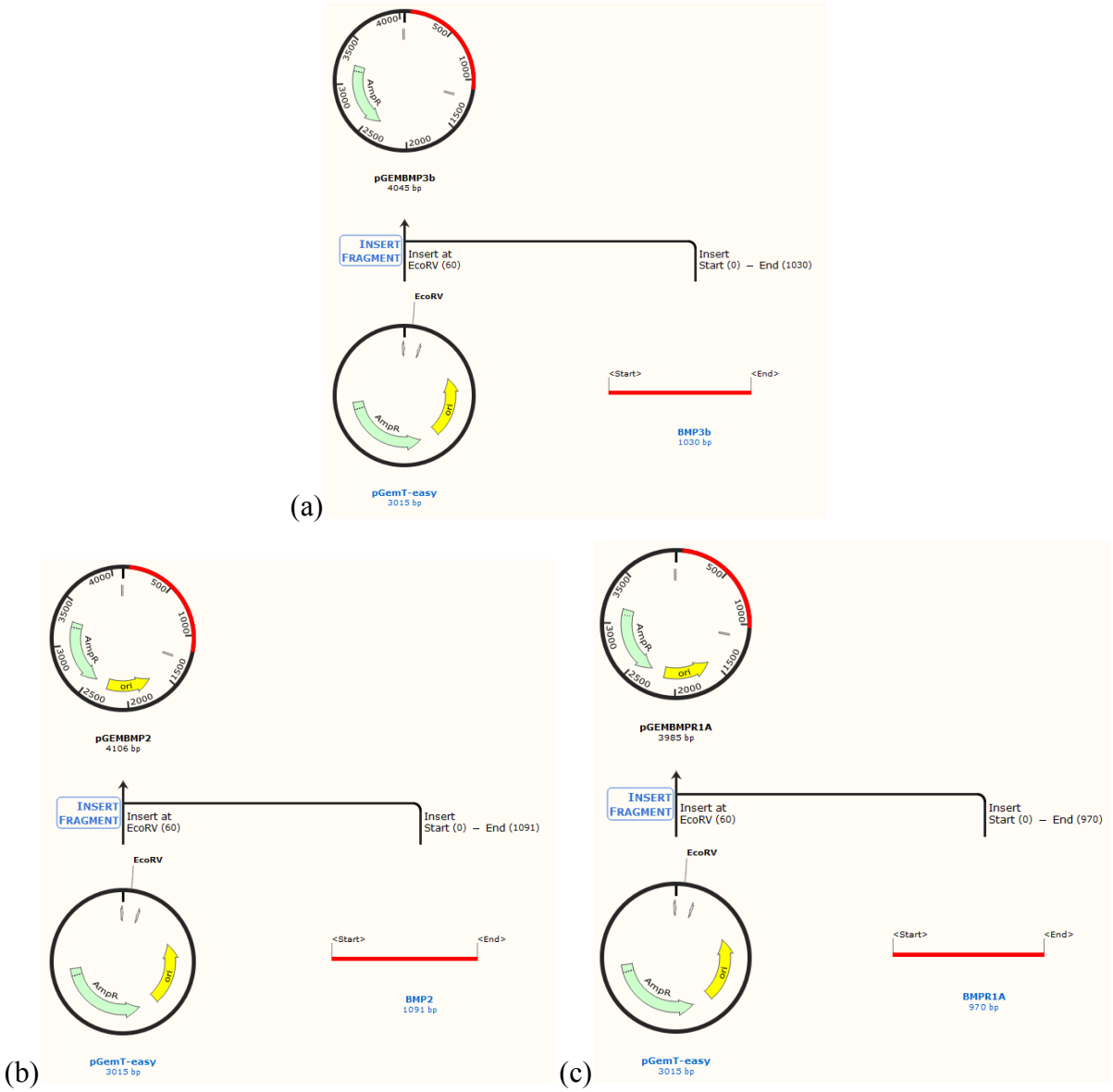


Figure 4.1 Cloning of *BMP3b, BMP2* and *BMPR1a* into pGEM-T Easy to create in situ hybridisation probes

cDNAs (approximately 1kb in length) for BMP3b (a), BMP2 (b) and BMPR1a (c) were created by PCR from H295R cDNA and cloned into pGEM-T Easy. Orientations were checked by restriction digest and the plasmids linearised with appropriate restriction enzymes targeting sites in the polylinker at the 5' end of the cDNA. Antisense ISH probes were synthesised using T7 or SP6 RNA polymerases as appropriate.

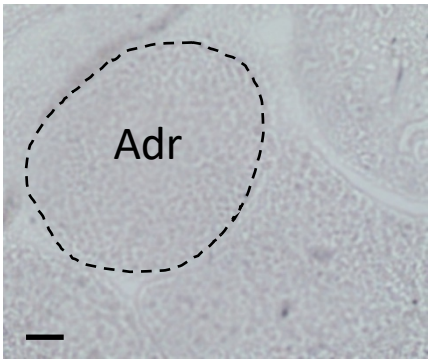


Figure 4.2 In situ hybridisation using *Bmp3b* sense probe - negative control
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 17.5 days, incubated with *Bmp3b* sense riboprobe as a negative control, resulting in no specific staining. The adrenal gland outlined by the black dotted line. Images were taken using the Leica DMR light microscope with 10x objectives. N=3, scale bar represents 200μm.

4.3 BMPs and adrenal development

To date there is no direct evidence linking BMPs to adrenal development. However, indirect data from the Booroola sheep, which have a naturally occurring mutation in *BMPR1B* coupled with a phenotype of significantly smaller adrenal glands (Souza et al., 2001), suggests this could be the case. Evidence of the presence and actions of BMPs within the adrenal cortex and medulla has been predominantly from *in vitro* studies using H295R and PC-12 cells respectively (Inagaki et al., 2006, 2007; Johnsen and Beuschlein, 2010; Rege et al., 2015). Within the adrenal medulla, there is *in vitro* and *in vivo* evidence showing that *BMP2, 4* and *7* are involved in the specification of sympathoadrenal cells (Schneider et al., 1999). Within the adrenal cortex, the data is mainly *in vitro*, showing BMP6 as a key factor to augment Ang II secretion and hence aldosterone production (Inagaki et al., 2006; Suzuki et al., 2004) and data from Johnsen found that BMP2 and BMP5 are able to suppress forskolin-induced steroidogenesis in H295R cells (Johnsen et al., 2009).

BMP3b has been documented to be involved in bone development and remodelling, hence vertebrae staining was used as a positive control to confirm the utility of the in situ probe generated. Non-radioactive ISH was carried out at different developmental stages of the male

Wistar rat - from E14.5 to adult - to identify when and where *Bmp3b* is expressed. The same analyses were performed for *Bmp2*, *Bmpr1a* and *Bmpr1b*.

4.4 Detection

4.4.1 BMP3b

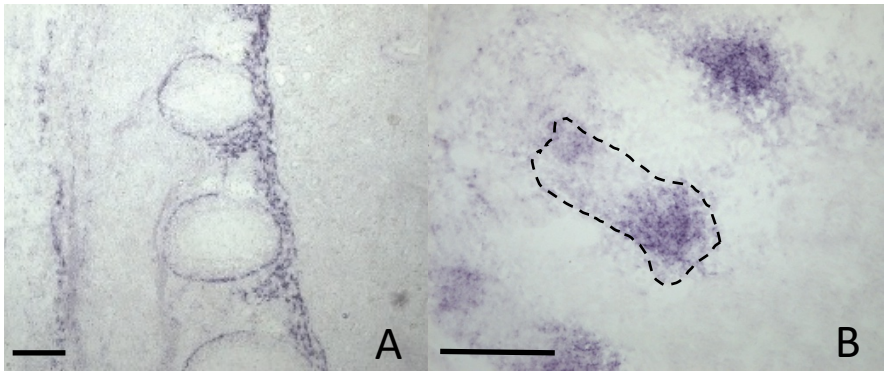


Figure 4.3 Positive *Bmp3b* mRNA staining in vertebrae - positive control
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 15.5 days, incubated with *Bmp3b* antisense riboprobe as a positive control, staining seen in vertebral bone. Images were taken using the Leica DMR light microscope with 10x (A) and 20x (B) objectives. N=3, scale bar represents 200µm.

At E14.5, no specific localised staining of *Bmp3b* in the adrenal gland/cortex was seen. However, by E15.5, there is some accumulation in parts of the subcapsular/zG area as shown in Figure 4.4. E17.5, *Bmp3b* mRNA is specifically localised to the subcapsular region and this staining remains into adulthood (Figure 4.5).

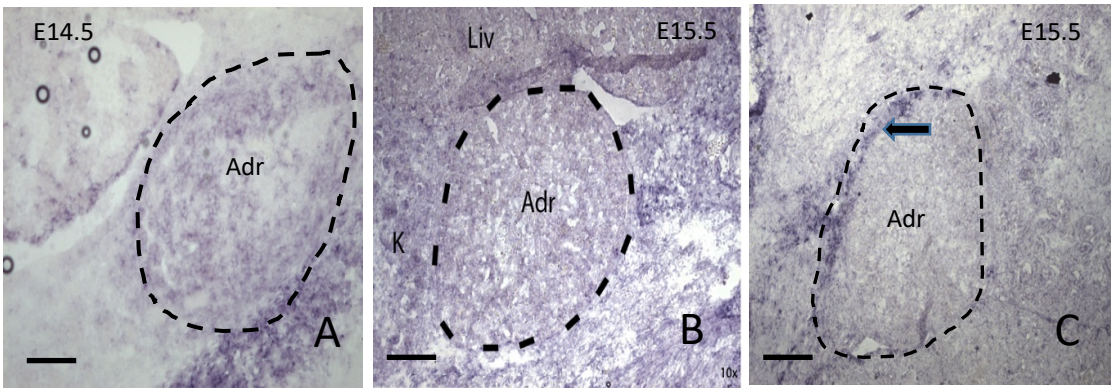


Figure 4.4 *In situ* hybridisation of *Bmp3b* in developing rat embyros
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 14.5 days (A) and 15.5 (B,C). They were incubated with Bmp3b antisense riboprobe in (A) and (C) and Bmp3b sense riboprobe in (B) as negative control. Images were taken using the Leica DMR light microscope with 10x objectives. N=3, scale bar represents 200µm. Black dotted line marks the adrenal gland. Arrow indicates staining around the capsular/zG boundary.

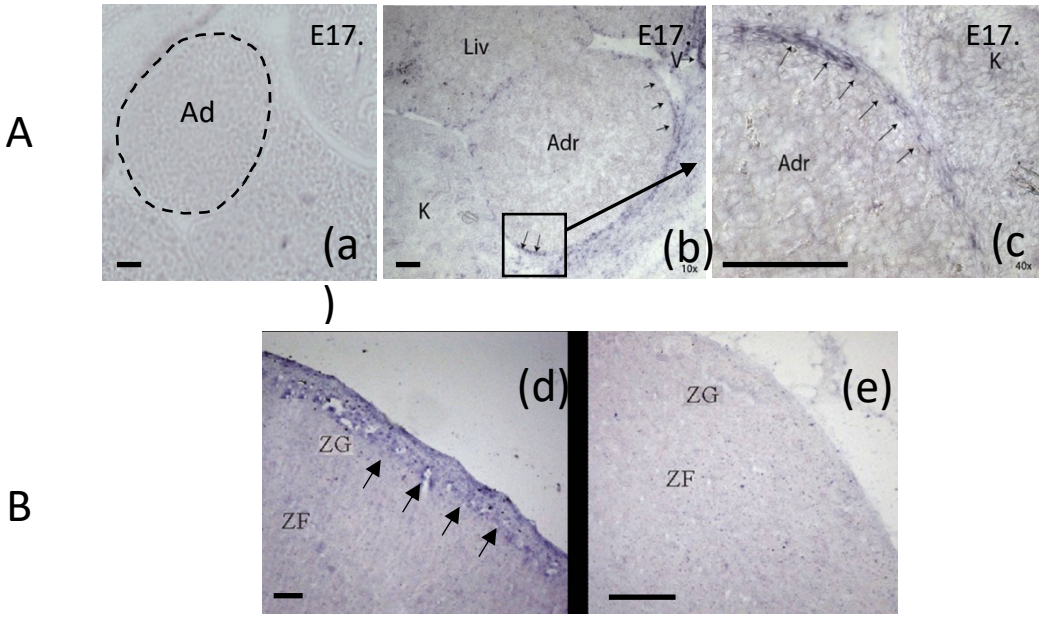


Figure 4.5 *In situ* hybridisation of *Bmp3b* staining is seen in rat adrenals
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 17.5 days (A) and adulthood (B). They were incubated with Bmp3b antisense riboprobe in (b,c,d) and Bmp3b sense riboprobe in (a,e) as negative control. Images were taken using the Leica DMR light microscope with 10x (a,b), 20x (d,e) and 40x (c) objectives. N=3, scale bar represents 200µm. Black dotted line marks the adrenal gland. Arrow indicates staining in the subcapsular region. V = vertebrae, K = kidney, Liv = liver, Adr = adrenal

Having established *Bmp3b* expression in the capsule of E17.5 rat adrenals, the expression of BMP receptors was investigated to identify the cells responding to BMP signals. BMP receptors can bind to any of the BMPs. Therefore, the presence of expression does not directly indicate an association with BMP3b. In Figure 4.6 below, definite staining of *Bmpr1a* was seen in the capsular region of the rat adrenal cortex by E15.5, 2 days earlier than the definite appearance of *Bmp3b*. The capsular staining remained throughout development (Figure 4.7). The staining of *Bmpr1a* was very uniform throughout the whole of the subcapsular region in contrast to *Bmp3b* staining that was patchy throughout the subcapsular region.

4.4.2 BMPR1A

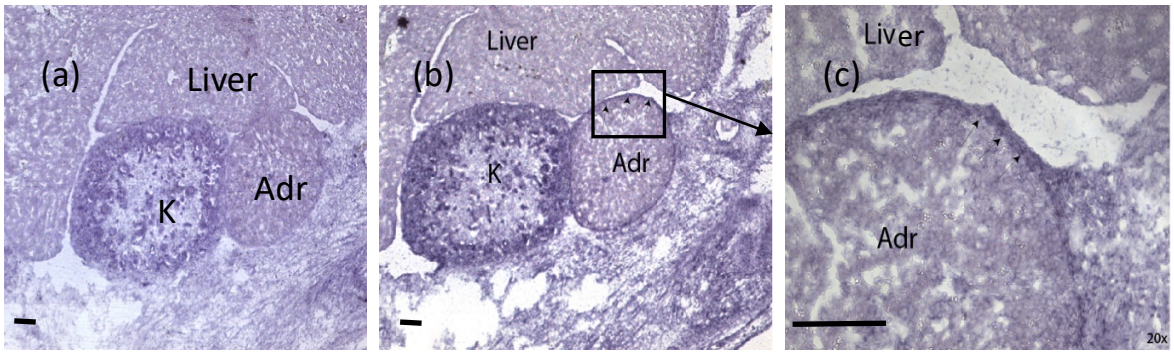


Figure 4.6 *In situ* hybridisation of *Bmpr1a* in rat adrenal at E15.5
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 15.5 days. They were incubated with Bmpr1a antisense riboprobe (b,c) and Bmpr1a sense riboprobe (a) as negative control. Images were taken using the Leica DMR light microscope with 5x (a,b) and 40x (c) objectives. N=3, scale bar represents 200µm. K = kidney, Adr = adrenal

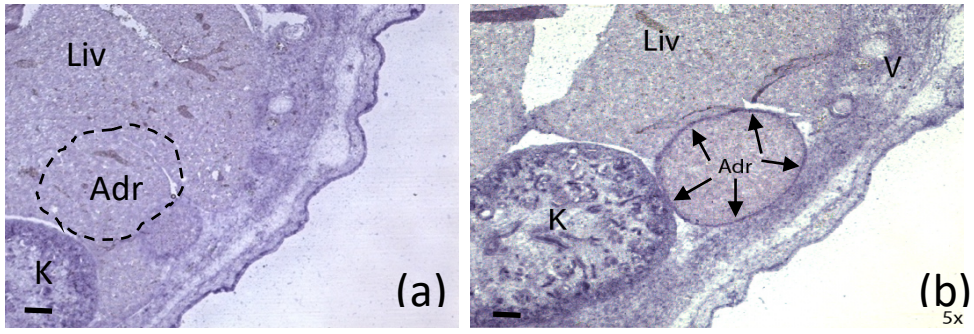
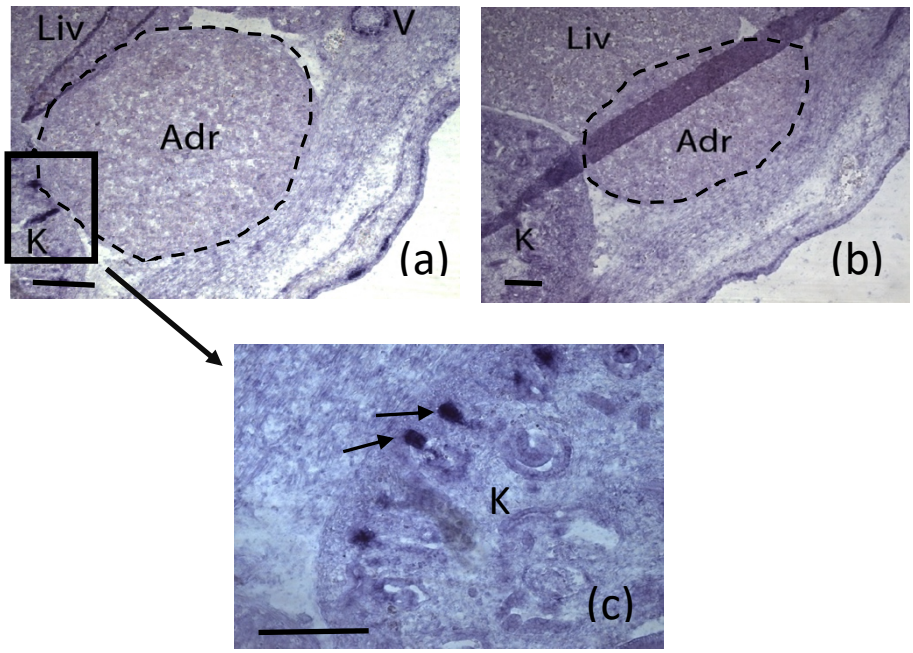


Figure 4.7 *In situ* hybridisation of Bmpr1a in rat adrenal at E17.5
Light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 17.5 days. They were incubated with Bmpr1a antisense riboprobe (b) and Bmpr1a sense riboprobe (a) as negative control. Images were taken using the Leica DMR light microscope with 5x objectives. N=3, scale bar represents 200µm. K = kidney, Liv = Liver, Adr = adrenal

4.4.3 BMP2 and BMPR1B

No staining was found with *Bmp2* and *Bmpr1b* probes in the adrenal cortex at any stage of development (Figure 4.8). This was a surprising result as previous studies have shown the presence of a mutation in *Bmpr1b* in Boorola sheep, which was linked to the observation of smaller adrenals (Souza et al., 2001). There was an absence of *Bmp2* staining in the rat adrenal cortex, which did not reflect the findings within the microarray and cell culture experiments performed here. This experiment was repeated 10 times with different probes made and *in situs* performed on samples from a wide range of embryonic stages. Positive staining was observed within the kidneys (Figure 4.8), which would confirm the lack of *Bmp2* staining rather than an issue with the probe. Therefore, immunofluorescence (IF) was performed with *Bmp2* to investigate its expression within the adrenal cortex (Figure 4.9). Using BMP2 antibody for IF, there was increased signalling within the inner cortex in the rat adrenal.



Bmp2 E17.5 Bmpr1b E17.5

Figure 4.8 In situ hybridisation of Bmp2 and Bmpr1b at E17.5
Representative light microscopy images of paraffin embedded adrenal sections from Wistar male rat, aged 17.5 days. They were incubated with *Bmp2* antisense riboprobe (a,c) and *Bmpr1b* antisense riboprobe (b). Images were taken using the Leica DMR light microscope with 5x (b), 10x (a) and 20x (c) objectives. N=6, scale bar represents 200µm. K = kidney, Liv = Liver, Adr = adrenal. Black arrows indicate renal tubules.

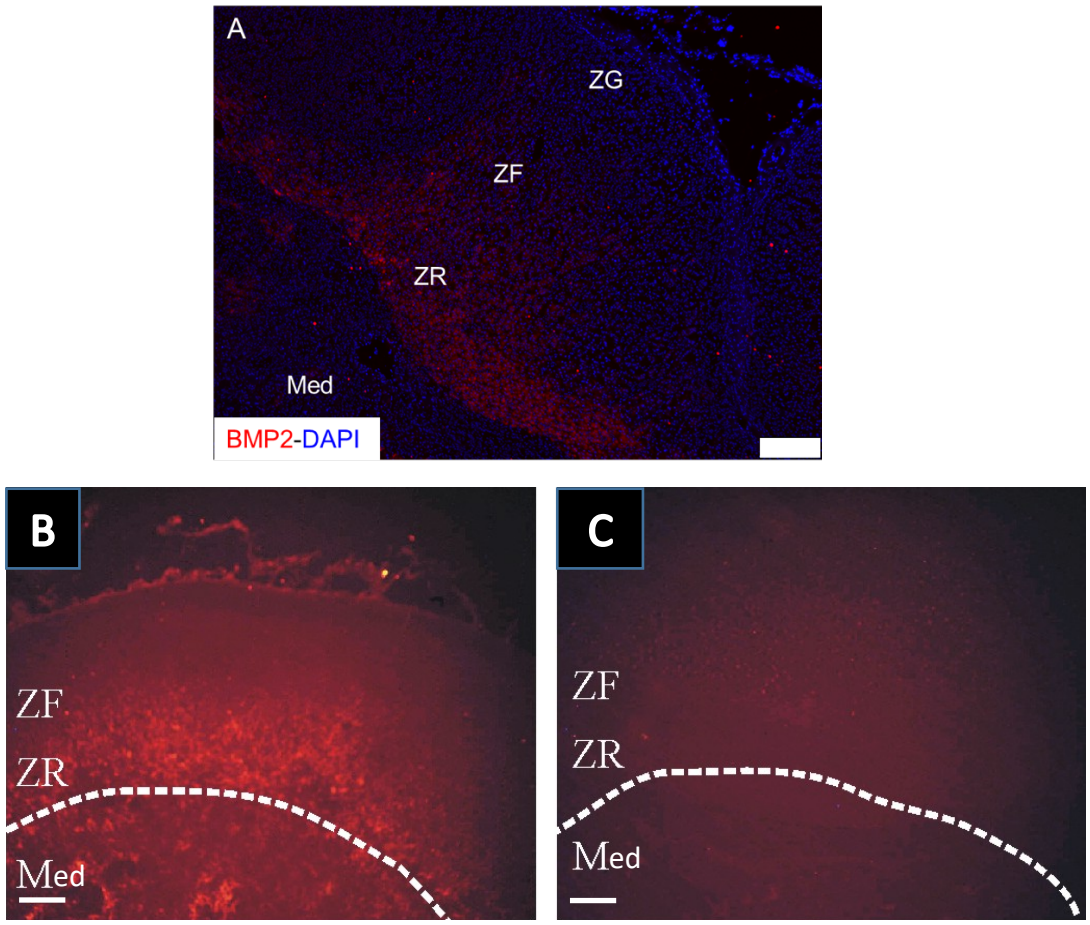


Figure 4.9 Immunofluorescence of BMP2 in adrenal gland
Immunofluorescence with BMP2 (red) and DAPI (blue) antibody using fresh frozen human adrenal gland (A) and adult Wistar rat adrenals (B). (C) shows immunofluorescence with only the use of secondary antibody (negative control). N=2, Scale bar 250µm. ZG = zona glomerulosa, ZF = zona fasciculata, ZR = zona reticularis, Med = medulla

1.

4.5 BMP3b knockout mice

Following the experiments localising mRNA for *Bmp3b*, *Bmp2* and its receptor *Bmpr1a*, *Bmp3b* knockout mice were obtained from Professor Lee (Zhao et al., 1999) as a kind gift. These were 8 weeks old *Bmp3b* KO CD-1 mice that were sacrificed on our behalf and the adrenal glands of male and female KO and WT mice were sent.

The knockout mice were generated with the deletion of the *Bmp3b* (GDF10) coding region by gene targeting. The gene consisted of 3 exons spreading over approximately 12 kb. The C-terminal domain, which is encoded in exons 2 and 3 was deleted and replaced by a neo cassette to ensure a full knockout was achieved (Figure 4.10). The F1 heterozygotes were crossed and produced offsprings that were wild types, heterozygous and homozygous in the ratio of 1:2:1. Northern analysis of RNA of the wild type, heterozygous and homozygous mice was performed and it showed complete RNA absence of *Bmp3b* in the homozygous mice. All the homozygous mice were viable and the appearances were reported to be grossly normal. The oestrous cycles of all the mice , pregnancy rate, gestation length and litter were no different (Zhao et al., 1999).

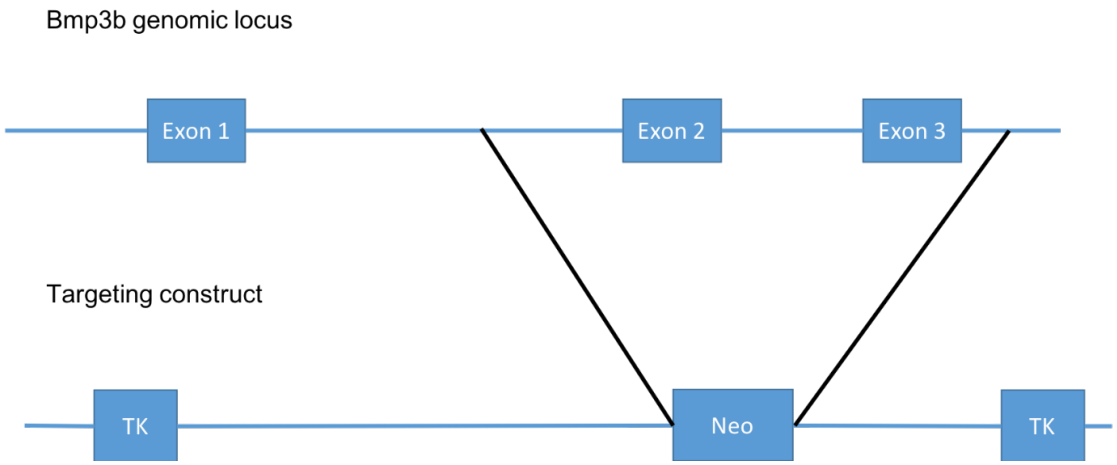


Figure 4.10 Diagramatic representation for creation of *Bmp3b* KO mouse
A Neo cassette was inserted to replace exon 2 and exon 3 to create a full length *Bmp3b* knockout model.

The mice adrenals received were fixed in PFA and transported in PBS. There were 3 WT and 3 KO mice adrenals sent for each sex. The adrenal glands were paraffin embedded and cut for immunohistochemistry (IHC) and IF.

The macroscopic appearance of the different zones of the adrenal cortex were similar in the wild type and knock out mice, when IHC was carried out using a CYP11B2 antibody specific to zG, which could be seen in the subcapsular region (Figure 4.11).

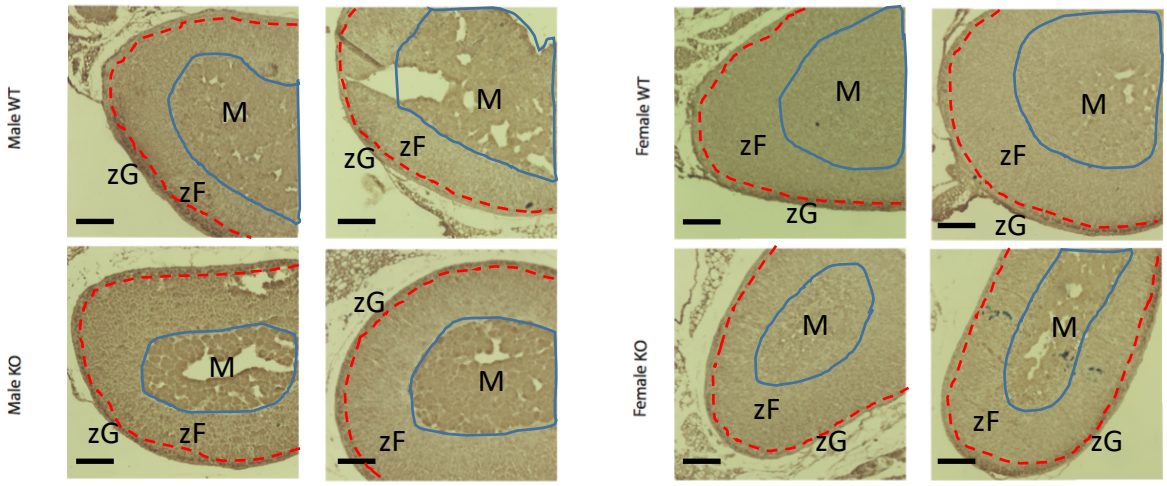


Figure 4.11 Cyp11b2 expression in *Bmp3b* knockout mice adrenals
Representative light microscopy images of paraffin embedded adrenal sections from wild type (WT) and *Bmp3b* knock out (KO) in both adult male and female mice. Immunohistochemistry were performed on adult male and female wild type and *Bmp3b* knockout mice adrenal glands, staining for Cyp11B2. Images were taken using the Leica DMR light microscope with 10x objectives. N=3, scale bar represents 200µm. Blue line indicates zona fasiculata (zF) and Medulla (M) boundary. Red dotted line represents zona glomerulosa (zG) and zF boundary.

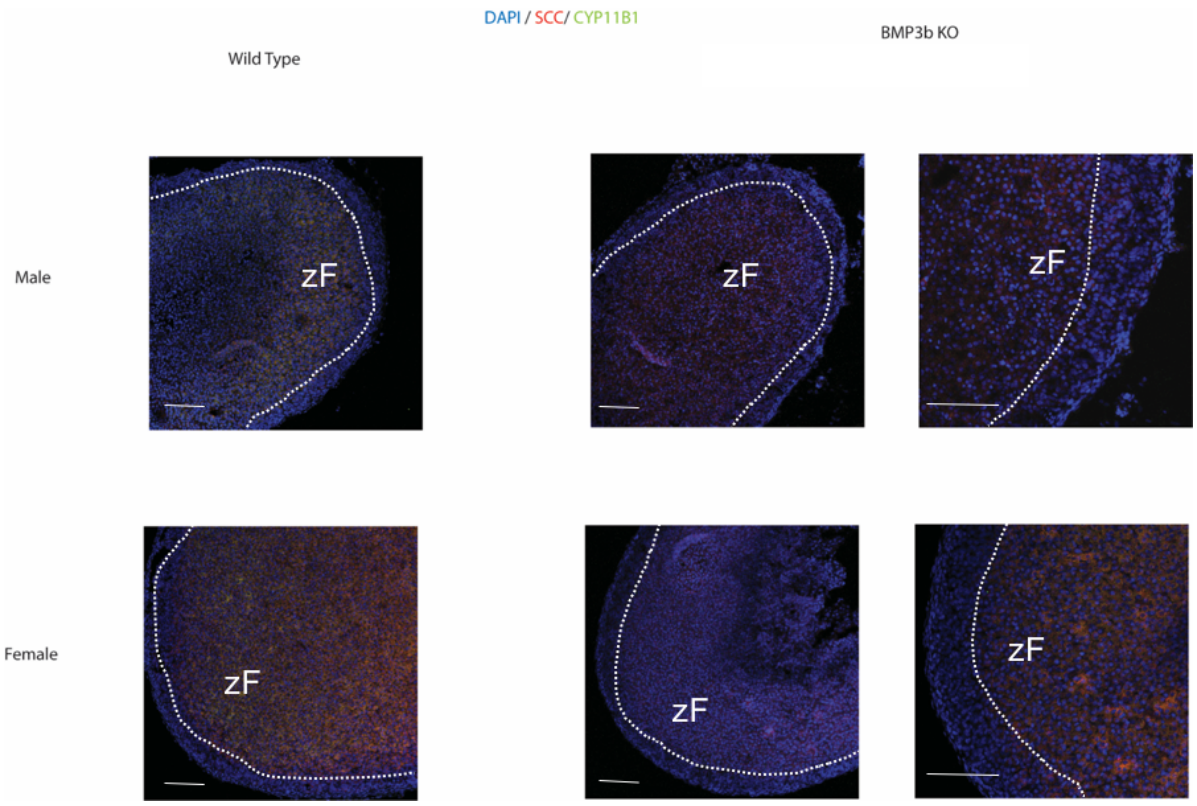


Figure 4.12 Immunofluorescence of mice adrenal glands for SCC and CYP11B1
Representative image of Immunofluorescence of mice adrenal glands: male and female wild types and *Bmp3b* KO. Nuclei are stained with DAPI (in blue), and antibodies against Side chain cleavage (SCC, in red and CYP11B1 in green) were used.
WT = wild type, KO = knockout

The comparison of the knockout and wild type mouse adrenals has shown that their anatomy and steroidogenic gene expression are very similar except in CYP11B1, where there appears to be a reduction in the knockout model compared to the WT (Figure 4.12). The reduction seen was not quantified and also may not be enough to affect steroidogenesis, hence *Bmp3b* KO mice remain viable.

4.6 Discussion

The histological studies have shown a preferential distribution of *Bmp3b* and *Bmpr1a* in the capsular region and *Bmp2* in the inner cortex. However, it was not possible to demonstrate the presence and location of many of the other BMPs, BMP receptors or antagonists (follistatin and noggin). This could be explained by the theory that interactions of the BMPs themselves are controlling the expression of each other, such as Bmp3b acting as an inhibitor of Bmp2 function as described in Chapter 3, hence an inhibitor, such as noggin, is not necessary. However, this theory would not fully explain why *Bmpr1a* is the only receptor expressed in the cortex. Therefore, with different BMPs being expressed in different areas of the adrenal cortex, the gradients they form may act to enhance or inhibit the delicate control of adrenal zonation and differentiation.

Bmp3b is present from E17.5 onwards in the rat adrenal, while *Bmpr1a* was detected from E15.5. Contrastingly, no expression of *Bmp2* or *Bmpr1b* was detected at any developmental stage by with in-situ hybridisation. We can detect *Bmp2* mRNA in the rat adrenal by qPCR, so this presumably indicates that the ISH was not sensitive enough and that levels of *Bmp2* were below the detection limits of our in situ assay. In the future, better antibodies or more sensitive ISH protocols may become available to give us a clearer understanding of the localisation of *Bmp2* and *Bmpr1b* mRNA within the adrenal gland. IF localisation of BMP2 showed increased staining within the inner cortex, towards the location of zR.

ISH has shown exquisite *Bmp3b* expression, supporting our microarray data of high *Bmp3b* in the 'capsular' fraction. The 'capsular' fraction is not exclusively capsular as the fracture plane of the adrenal gland will inevitably be contaminated by some zF cells within the majority of zG and capsular cells (approximately 5%, as suggested by personal communication with Prof Gavin Vinson, QMUL). However, even though the inner fraction may contain some zG cells adjacent to the fracture plane, it is highly unlikely that there would be any capsular content, and hence no *Bmp3b* expression was detected in the inner cortex fraction. Given the specific capsular location of *Bmp3b* expression, it is possible to speculate that it may play a role in stem cell and zonation of the adrenal cortex and may even have interplay with other important genes

that are known to be involved in stem cell development and maintenance with the cortex, such as *Shh* and *Rspo3* (Guasti et al., 2010; Vidal et al., 2016). *Rspo3* is expressed in the capsule, and these cells are the target of Shh signalling from the cortex, and lineage tracing studies have shown that capsule cells can differentiate into cortical cells both during development and in the adult organ. The effect of Shh and/or Wnt signalling on *Bmp3b* expression, and vice versa, would be interesting to define. Other studies have shown interplay between SHH and BMP, such as in human haematopoietic cells (Bhardwaj et al., 2001) and co-localisation in other tissue such as teeth, hair and gut (Bitgood and McMahon, 1995). Rspo3 has been shown to determine and maintain glomerulosa cell fate throughout life by inducing *Wnt4* and *Shh* expression (Vidal et al., 2016). Therefore, *Bmp3b* may also have a role in the determination and maintenance of glomerulosa cell fate as the location of the gene makes it plausible that they could interact, though this has not yet been shown. Rspo3 could act as the main driver of glomerular cell fate via Wnt and Shh, whilst Bmp3b could be the opposing factor to control or fine tune the cell's fate, similar to our proposed theory of BMP2 and BMP3b in Figure 3.15, which would require further studies to show this potential interaction.

However, the contribution of *Bmp3b* to glomerular cell fate may be small as the KO mouse models used in this work are all viable. Therefore the role Bmp3b plays may be more towards stem cell behaviour or fine tuning of its function and would be useful to investigate the function of the adrenal gland in aging KO mice, as the stem cells deplete.

Naturally occurring mutations in *BMP3b* have not been found in humans but the *Bmp3b* null mouse does survive, suggesting that BMP3b is unlikely to be crucial for control of the adrenal stem cell niche or adrenal zonation maintenance. However, the adrenal glands of these mice have not been fully looked at in published data. Therefore, using *Bmp3b* null mice in these experiments were useful to give a more detailed insight into the role of *Bmp3b* in adrenal morphology. Given that the *Bmp3b* knock out mice survive, this indicates that *Bmp3b* cannot be essential adrenal function, which is known to be vital. On comparing the wild type to *Bmp3b* KO mice, the size of the adrenals (although they were not weighed) and the cortical anatomy appeared similar, though IF suggests that there was less signal for Cyp11b1 and Scc but the IHC for Cyp11b2 was very similar in staining. This suggests that there may be a

reduction in steroidogenesis in *Bmp3b* KO mice, but not enough for it to be fatal if *Bmp3b* was not present. This result is inconsistent with the *in vitro* data which suggest that BMP3b is inhibitory to *CYP11B1* expression, at least in H295R cells. The mouse adrenals were not re-genotyped on receipt and it remains formally possible that they were mislabelled by our collaborators. However, they have assured us that this has never happened before and is not a likely scenario. However, the increased expression of *BMP2* across the cortex towards the medulla in both rats and humans does support the model in the previous chapter which hypothesises that *BMP3b*, expressed in the capsule, is inhibitory to *BMP2* expression in the outer region of the cortex, and BMP2 exerts its most striking effect on enhancing steroidogenesis in the zR.

According to the H295R results, effects of BMP2 that are modulated by BMP3b were mainly on androgen expression and these studies were carried out on mice, which do not produce androgens Therefore, the mice model may not have been the most appropriate system for this study hence the results we had expected were not observed. It is probably more appropriate to concentrate on expression studies in human adrenals, looking at expression of *BMP3b, BMP2* with the addition of *SHH, WNT4* and *RSPO3* within the adrenal cortex for potential co-localisation.

Limitations to these studies were present. The ISH protocols have been altered many times to optimise detection but this did run the risk of over exposure and an increase in backgound noise. ISH or IF expression were not quantified in the experiments and the studies were thus purely qualitative. This means I am only able to comment subjectively on the presence or absence of staining relative to negative and positive controls, so any subtle differencs in gene expression could have been overlooked. In the study, it would have been ideal to confirm concordant expression using both ISH and IF techniques but this was not possible as the BMP3b antibody did not work for IF and *BMP2* ISH was not concordant with IF findings, therefore further experiments would be needed to confirm the presence or absence of BMP2. The study could have been improved by the use of multiple labelling of the tissues to test for expression or co-expression of BMPs and their receptors simultaneously. Other techniques could also have been considered; such as radioactive ISH, but this option was not used due to

the lack of a radioisotope facility and this remains a subjective measure of expression. IHC would have been another option but the BMP antibodies were not reliable enough to ensure that any lack of expression was a true result or because of the ineffective antibody. A new technology known as RNAscope would have been a good alternative method as it has been shown to reduce signal to noise ratio as it has a more specific RNA binding of two probes that are aligned next to each other before a signal is produced. This method is able to quantify RNA that is anatomy specific and would be useful in the studies (Wang et al., 2012a).

This study used normal rat adrenal glands sacrificed for other studies to gather initial information on RNA expression of *Bmp3b* and *Bmp2* within the adrenal cortex. However, this was not able to demonstrate the direct effect of *Bmp3b* and *Bmp2* within the cortex. This could have been demonstrated by manipulation of Bmp3b and Bmp2 within the cortex of rats. This was attempted as part of the experiments where Bmp3b and Bmp2 soaked Affi-gel beads were inserted into the rat adrenals but the attempt was unsuccessful in obtaining rat adrenals with Affi-gel beads still in place to be observed and studied.

5 Is BMP3B involved in adrenal tumourigenesis?

5.1 Aim

During the course of this study tissue was collected from adrenalectomies performed at Bart's and the London and other London hospitals between September 2008 to September 2011. The tissues collected were from a number of different adrenal diseases along with adjacent normal adrenal tissue Table 5.1. The ethical approval for adrenal tissue collection was granted by Redbridge and Waltham Forest Local Research ethics Committee on 8th August 2008. REC reference number: 08/H0701/59 'Analysis of bone morphogenetic protein and sonic hedgehog signal transduction pathway components in adrenal tumours and adjacent normal tissue'.

The aim was to investigate if BMP3b and other BMPs and Shh are biological markers of adrenal cortical disease: Adrenocortical carcinoma and APAs. The prognosis for Adrenocortical carcinoma (ACC) remains poor over the years with no improvement in treatment modality or survival. Although APAs are benign, the consequences of hyperaldosteronism can cause severe cardiovascular morbidity.

5.2 Biological markers

Biological markers for adrenal disease would be extremely helpful in predicting prognosis and response to treatment. In ACC, the current biological markers of disease would be steroid secretion in up to 75% of cases (Abiven et al., 2006). Usually a combination of steroid hormones are secreted, unlike benign tumours which mostly secrete one type. Cortisol and androgens are most frequently secreted (Table 5.2) (Luton et al., 1990) but ACC is also capable of secreting mineralocorticoids and steroid precursors, which can be detected in urine (Arlt et al., 2011). Steroid measurements help with guidance of disease activity in patients but cannot predict prognosis or treatment response. Therefore, new biological markers that answer these questions would be extremely useful, so that patients are provided with more knowledge and treatment could potentially be tailored to each individual patient. However, a biological marker must be robust: easily measurable, preferably from patient's blood or urine, specific to

the disease and cost effective (Strimbu and Tavel, 2010). Over the last decade, there has been work on microRNA (miRNA) as potential biological markers as they can be detected in the blood stream and also as a potential therapeutic target. MicroRNAs are small, non-coding RNAs of 18-24 nucleotides in length that are highly conserved. Their role is to silence target genes by degrading mRNA or inhibiting translation (Ambros, 2004; Cherradi, 2015; MacFarlane and Murphy, 2010). Over 20 miRNAs has been associated with ACC, with a handful of them validated in patients, such as miR-483-5p, miR-100, miR-34a, miR-195 and miR-335 (Chabre et al., 2013; Patel et al., 2013; Szabó et al., 2014, 2013).

5.3 Hyperaldosteronism

The two most common subtypes of hyperaldosteronism are APAs and BAH. Treatment is vastly different in these two types of hyperaldosteronism: APA can be treated with an adrenalectomy, whereas medical therapy is the treatment of choice for BAH. Currently, diagnosing APA or BAH would require biochemical testing, imaging and invasive adrenal vein catheterisation. Therefore, a biological marker that is able to identify the different subtypes would be extremely helpful for diagnosis and treatment of patients. Attempts have been made by many groups to distinguish between the two sets of patients, with postural aldosterone levels which were not very specific or sensitive enough to distinguish between the two (Lau et al., 2012) or the use of urinary 18-hydroxycortisol (18-OHF) which is increased in APAs, though the measurement of 18-OHF is not readily available (Miyamori et al., 1992). Currently, there is no published data on miRNA as biological markers for primary aldosteronism nor any other biological markers available.

During the course of this PhD, Shh was suggested to be a marker of stem/progenitor cells in the adrenal cortex, and numerous studies had shown aberrant signalling to be a feature of several types of cancer and to be involved in the maintenance of cancer stem cells (Cochrane et al., 2015; Dierks et al., 2008; Kelleher, 2011; Lau et al., 2006; Song et al., 2011; Watkins et al., 2003). Guasti *et al.,* showed that in the rat Dlk1 (Delta-like 1 homolog), well documented as a negative regulator of adipogenesis (where it has been referred to as Pref-1), and has been

suggested to play a similar role in the adrenal (Guasti et al., 2013b), is co-expressed with *Shh* and can modulate its signalling. Dlk1 has also been suggested to be a marker of cancer stem cells in hepatocellular carcinoma (Xu et al., 2012). It was therefore decided to investigate the expression of these markers and other pathway components in the adrenal tissues, as well as BMPs, the main focus of this thesis so far. BMPs are involved in organ development and in humans have been implicated in many tumour processes (Beck et al., 2006; Blanco Calvo et al., 2009; Huang and Klein, 2004). Studies by Beuschlein's group have already shown that *BMP2* and *BMP5* expression are suppressed in adrenocortical carcinomas compared to normal adrenal samples (Johnsen et al., 2009). This project so far has shown that BMP3b has a suppressive effect on BMP2 *in vitro*, and this has been supported by the gradient of expression observed in the *in vivo* study. Given the evidence that BMP2 is present in adrenal tumours (Johnsen et al., 2009), BMP3b expression in adrenal adenomas and carcinomas was investigated. Furthermore, the Shh and BMP pathways show clear interplay (Bitgood and McMahon, 1995), such as in limb patterning (Dahn, 2000; Drossopoulou et al., 2000), hematopoietic cell proliferation (Bhardwaj et al., 2001; Bitgood and McMahon, 1995) and osteoblast differentiation (Zhao et al., 2006). The hedgehog pathway itself also plays a role in many cancers. For example, Shh is upregulated in dysplastic cells in colorectal cancer (Bian et al., 2007), activation of the pathway through patched mutation in basal cell carcinomas (Bale, 2001) and increased expression of hedgehog pathway component target genes, *Gli1* and *Ptch* is seen in gastric cancer stem cells (Song et al., 2011).

5.4 Adrenal samples

Adrenal Tissue Type	Numbers collected
Adjacent normal tissue	13
Aldosterone producing adenomas	12
Adrenocortical carcinomas	4
Adrenal hyperplasia from Cushing's disease	5
Phaeochromocytoma	2

Table 5.1 Types of adrenocortical tissues collected between 2008-2010 at St. Bartholomew's Hospital

Tumour Type	Average 9am cortisol level (nmol/L) Normal 200-600nmol/L	Average recumbant aldosterone level (pmol/L) Normal 135-400pmol/L
Aldosterone producing adenoma	239	907
Adrenocortical carcinomas	575	162
Adrenal hyperplasia from Cushing's disease*	1245	N/A

Table 5.2 Average level of steroid production in patient cohort
*N/A – not done as part of workup, * bilateral adrenalectomy for Cushing's disease*

5.5 Real time qPCR of adrenal tissue

5.5.1 Zone specific expression in adrenal tissue

The first experiments were performed to confirm the presence and expression of *CYP11B1* representing zF and *CYP11B2* representing zG in normal tissue compared with adrenal tumours of different aetiology. It is expected that APAs would express the highest level of *CYP11B2* expression in the adenoma, as in other published data (Enberg et al., 2004; Nakamuraa et al., 2014). The results, shown in Figure 5.1 showed a significant reduction *CYP11B1* expression in APAs ($p<0.05$) and phaeochromocytoma ($p<0.01$) patients compared to normal, which would be expected as neither of the two conditions are known to produce cortisol. However, there were no significant difference between adjacent normal tissue and Adrenocortical carcinomas or hyperplastic samples, where a 2-fold and 6-fold increase in cortisol production respectively was measured in the patient's serum cortisol. However, the 'normal' used was adjacent normal of APAs and the expression may not be the same as a truly normal adrenal gland, which could not be obtained as this was not part of the ethical approval.

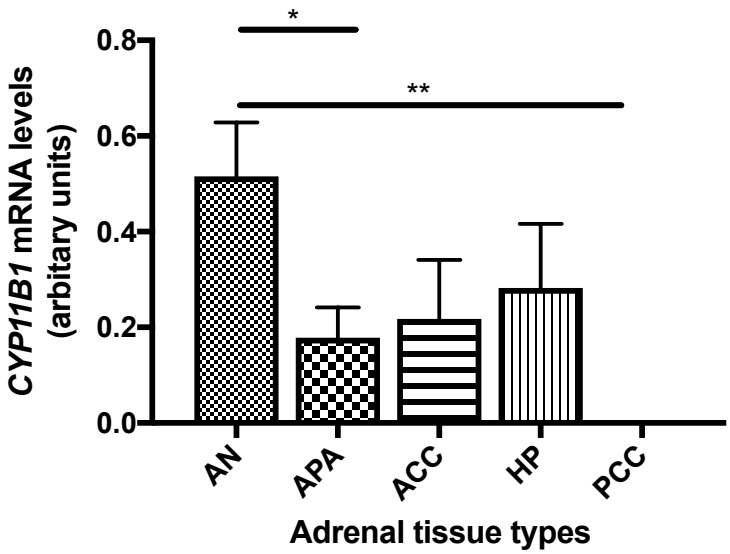


Figure 5.1 *CYP11B1* expression levels in different types of human adrenal tissue
Comparison of *CYP11B1* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM. *p<0.05, **p<0.01, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

With *CYP11B2*, expression level was as predicted with significantly increased *CYP11B2* expression ($p<0.001$) correlating with increased aldosterone production in APAs (Figure 5.2). There may also be a confounding factor that the normal tissues used in this study is adjacent normal, hence there may be more suppression of *CYP11B2* expression than truly normal adrenal tissue. The mRNA expression data showed no *CYP11B1* or *CYP11B2* expression in phaeochromocytoma samples, which are tumours of the adrenal medulla, where there should not be any cortisol or aldosterone production.

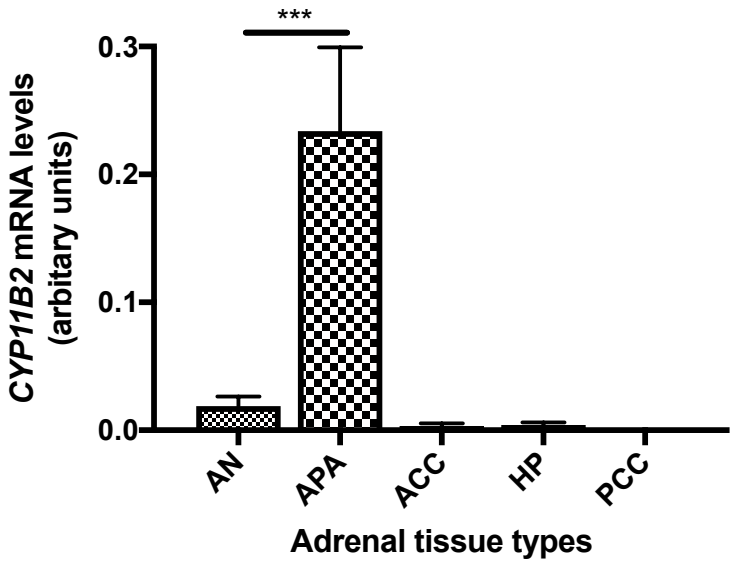


Figure 5.2 *CYP11B2* expression levels in different types of adrenal tissue
Comparison of *CYP11B2* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
***p<0.001, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

Following on from the above findings, *DLK1*, which plays a role in the maintenance of mesenchymal stem/progenitor cells (Kim et al., 2009; Lin and Yun, 2010; Sul et al., 2000), is only found in the subcapsular region of the adrenal cortex and the medulla (Guasti et al., 2013b; Raza et al., 1998). With *DLK1* being found in the subcapsular region, where progenitor cells are thought to reside, its presence in the samples may indicate an increase in progenitor cells. This real time qPCR (Figure 5.3) showed that *DLK1* was present in the medulla, as expected but its expression in the other adrenal tissues was not significantly different from normal adrenal tissue. This may be explained by the low expression of *DLK1* in the inner cortex, which is included in the sample and not all samples definitely included the subcapsular fraction.

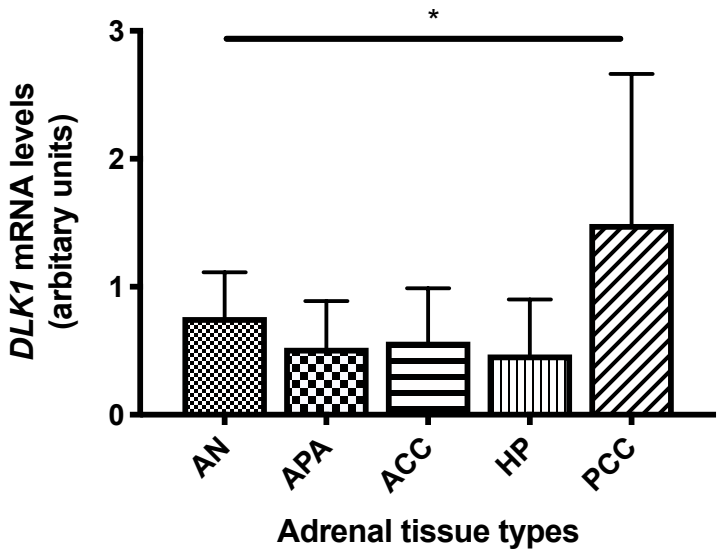


Figure 5.3 *DLK1* expression in different types of adrenal tissue
Comparison of *DLK1* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
*p<0.05, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

5.5.2 Sonic hedgehog and *GLI1* expression

The next experiments looked at *SHH* and *GLI1* expression. *SHH* has been implicated in many cancer processes such as basal cell carcinoma, prostate and pancreas (Chen et al., 2011; Datta and Datta, 2006; Lau et al., 2006; Lupi, 2007) and *Shh* plays an important role in development (King et al., 2008). *Gli1* expression is dependent on canonical activation of the Hh pathway and so *Gli1* is used in many experiments as a marker for active *Hh* signalling (Vokes et al., 2007).

The expression of both *SHH* (Figure 5.4) and *GLI1* (Figure 5.5) was reduced compared to normal. These results indicate that *SHH* and its pathway components are present in both normal and abnormal adrenal cortex. The expression of *SHH* does not vary significantly across the adrenocortical groups though there was also a large variance in the normal sample, which may have affected the statistics. However, there was significantly reduced levels of *GLI1* in the ACC and there was also a significant decrease of *GLI1* in ACC compared to APAs. *SHH*

levels in PCC are very low, which could be background levels as *SHH* is not found in the medulla. However, there was an increase in expression of *GLI1*, which cannot be explained by *SHH* presence. There is no published data on *IHH* or *DHH* hedgehog in PCC to account for the *GLI1* expression. However, in von Hippel Lindau syndrome, where patients can develop phaeochromocytomas, a mutation in the VHL gene is present. VHL is a tumour suppressor gene that decreases *GLI1* expression. Therefore, a mutant gene would conversely allow *GLI1* expression (Cho et al., 2013).

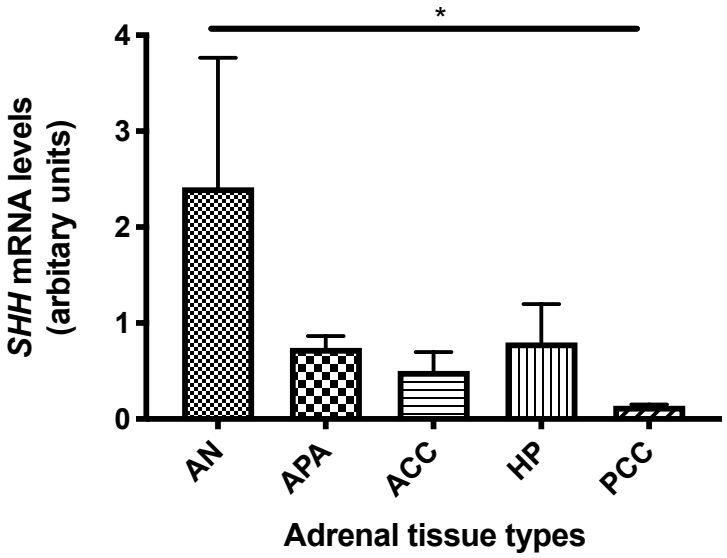


Figure 5.4 *SHH* expression in different types of adrenal tissue
Comparison of *SHH* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
*$p<0.05$, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

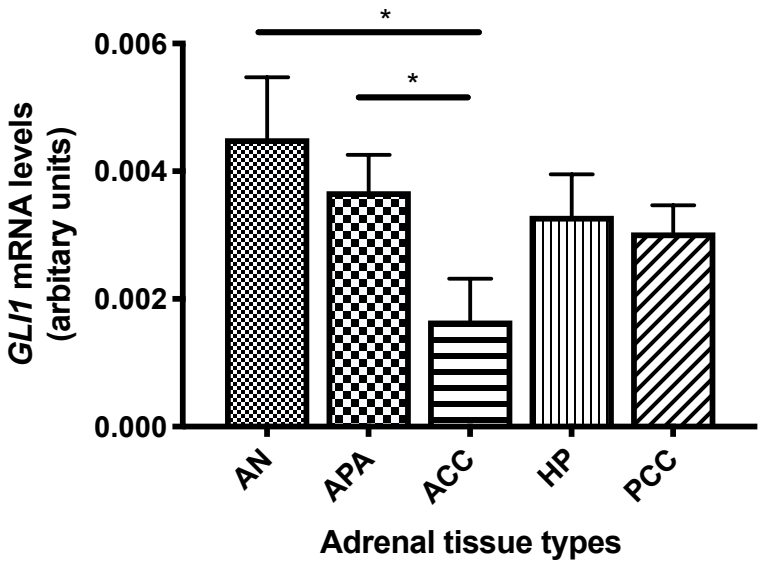


Figure 5.5 *GLI1* expression in different types of adrenal tissue
Comparison of *GLI1* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
*p<0.05, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

5.6 BMP expression

Evidence of *BMP* expression in the H295R cells as shown in Chapter 3 led to the investigation of their expression in human adrenal tissue. The BMPs investigated were those known to be present in the adrenal cortex or H295R cells. The *in vitro* studies have shown that *BMP2* and *BMP3b* are present in the adrenal cortex (H295R cells), other published data confirms presence of *BMP6* (Inagaki et al., 2007; Otani et al., 2010), *BMP2* (Johnsen et al., 2009) and *BMP4* (Rege et al., 2015).

BMP2 expression was not significantly lower in all the abnormal adrenal tissues, except the hyperplastic samples (Figure 5.6). The reduced expression of *BMP2* in ACC found in our cohort was not found to be significant, which may be due to the small number of samples used or the high variance in the normal samples, hence this experiment could not replicated the

results published by the Beuschlein group (Johnsen et al., 2009). There was not a significant reduction in APAs, possibly due to very little *BMP2* expression in the outer cortex. Though the histology of an APA can have zG, zF or zR phenotypes, the most common phenotype seen in our cohort was zF and this is consistent with other published data. Despite the zF like phenotype, the cells are still thought to express *CYP11B2* (Boulkroun et al., 2010, 2011; Nishimoto et al., 2010).

BMP4 is expressed at higher levels in the zG than the inner cortex, even though it exerts its effects on C19 synthesis (Rege et al., 2015), but it did not show any significant difference in expression with APA or adrenocortical carcinoma patients (Figure 5.7). However, there was a significant reduction in phaeochromocytoma samples. *BMP4* has not been shown in other studies to be present in the adrenal medulla and this could explain this significantly low expression in phaeochromocytomas, which is likely to be background levels.

BMP6 is involved in the induction of aldosterone production (Matsumoto et al., 2012). The real time qPCR results did not show any significant difference in *BMP6* expression in normal and abnormal adrenal tissue (Figure 5.8). *BMP6* is known to augment the production of aldosterone in adrenocortical cell lines in response to angiotensin II treatment (Suzuki et al., 2004) and its lack of significant expression compared to normal tissue may indicate that *in vivo*, *BMP6* may not have a major role in aldosterone production in APAs. The phenotype of the APAs could have affected *BMP6* expression due to the predominance of zF like cells and that adjacent 'normal' zG has been shown to express reduced levels of *CYP11B2* in the presence of an adenoma (Boulkroun et al., 2010).

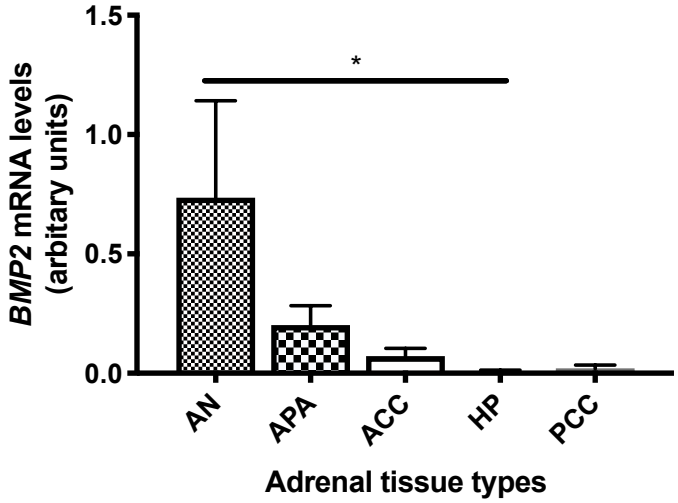


Figure 5.6 *BMP2* expression in different types of adrenal tissue
Comparison of *BMP2* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
*p<0.05, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

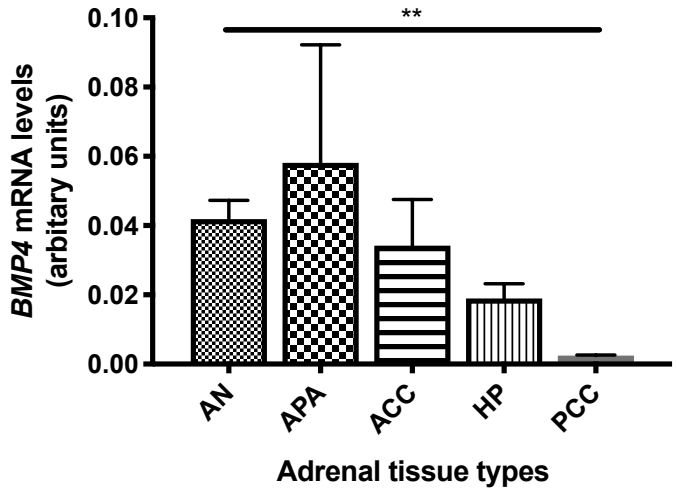


Figure 5.7 *BMP4* expression in different types of adrenal tissue
Comparison of *BMP4* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
**p<0.01, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

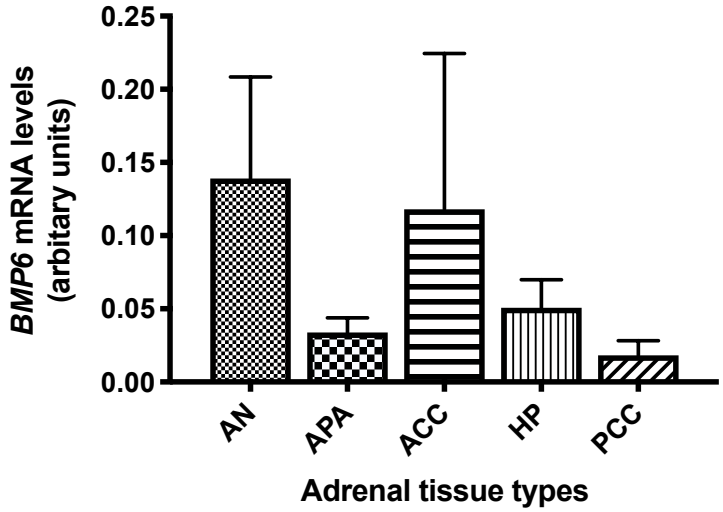


Figure 5.8 *BMP6* expression in different types of adrenal tissue
Comparison of *BMP6* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
One way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

5.6.1 *BMP3b* expression

BMP3b, expressed solely in the adrenal capsule, was investigated next. It would be predicted that *BMP3b* would increase in expression, if *BMP2* was downregulated in these samples (though our original samples did not reach significant downregulation of *BMP2*). The figures below showed *BMP3b* expression both within our study cohort (Figure 5.9, Figure 5.10, Figure 5.11) and also from a German cohort (Figure 5.12) who had published data on the downregulation of *BMP2* and *BMP5* in adrenocortical carcinoma (Johnsen et al., 2009).

There was a subjective difference in *BMP3b* expression in the 4 individual adrenocortical carcinoma cases, where 2 had lower expression and 2 with higher expression. With *BMP2* expression, there were no significant difference in *BMP3b* and *BMP2* levels.

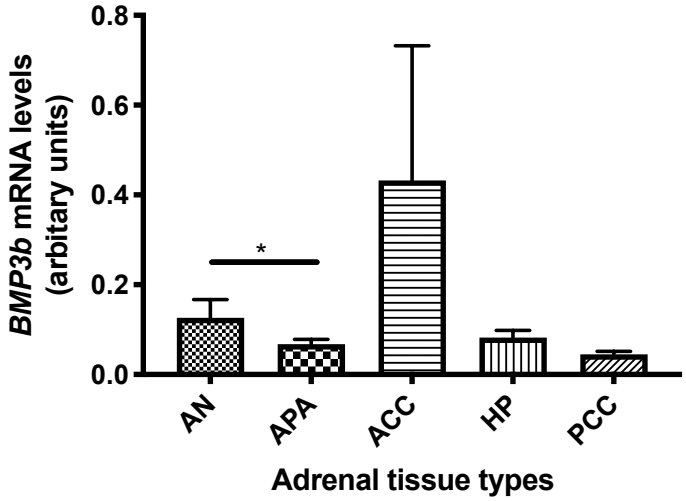


Figure 5.9 *BMP3b* expression in different types of adrenal tissue
Comparison of *BMP3b* expression in different types of adrenal tumours against their adjacent normal adrenal tissue. N=3, performed in triplicates. Error bars indicate SEM.
*p<0.05, one way ANOVA used.
AN=Adjacent normal adrenal tissue, APA=Aldosterone producing adenoma, ACC=adrenocortical carcinoma, HP=Hyperplasia, PCC=phaeochromocytoma

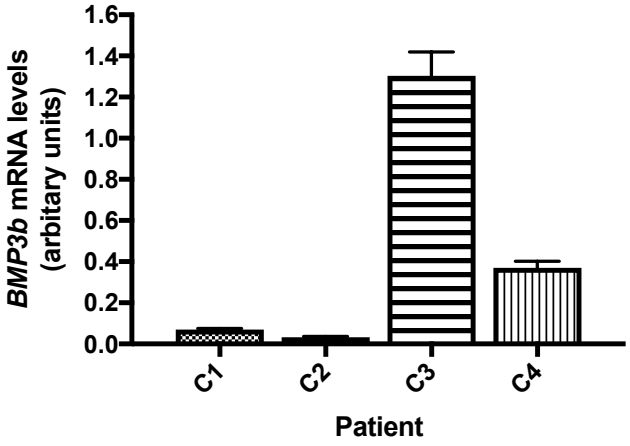


Figure 5.10 *BMP3b* expression in each adrenocortical carcinoma patient
BMP3b expression in individual adrenocortical carcinoma patients, represented by C1-C4. N=1, performed in triplicates. Error bars indicate SEM.

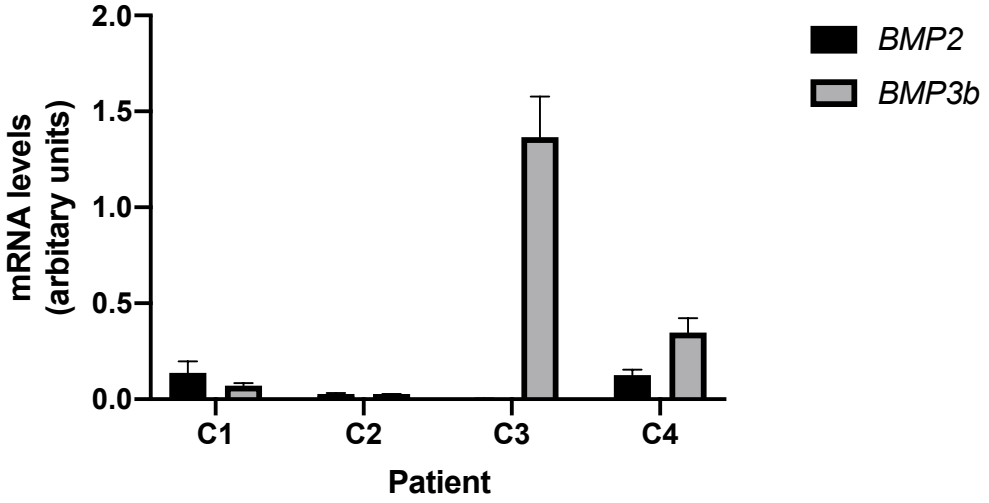


Figure 5.11 *BMP3b* and *BMP2* expression levels in each adrenocortical carcinoma patient
Comparison of *BMP3b* and *BMP2* expression in individual patients.
N=1, performed in triplicates. Error bars indicate SEM.

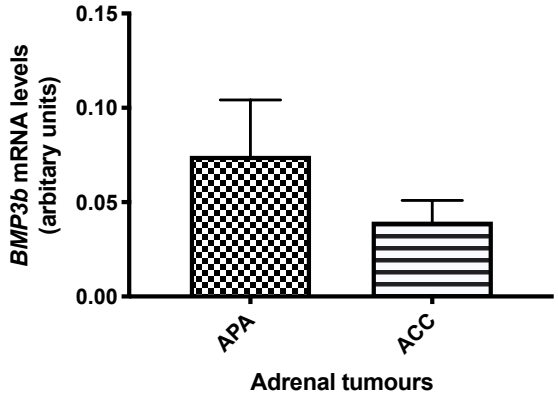


Figure 5.12 *BMP3b* expression in adrenal tumours in a German cohort
Comparison of *BMP3b* expression in APA and ACC. N=3, performed in triplicates. Error bars indicate SEM. Student t-test used.
APA = aldosterone producing adenoma, ACC = adrenocortical carcinoma

The UK and German cohorts did differ in their *BMP3b* expression. The UK cohort was very consistent amongst the APAs, whereas the adrenocortical carcinomas showed an overall upregulation but this was due to high expression in 2 out of the 4 ACCs, rather than all 4. On

reviewing the clinical details of the 4 ACCs (Table 5.3), the 2 patients with the lowest grade of tumour on histology had the highest *BMP3b* expression and the one with the most suppressed expression was the most aggressive tumour histologically, though has survived beyond 5 years as all of these patients only had local disease. The clinical details of the German cohort were not obtained hence conclusions cannot be drawn with clinical relevance.

	Age/ Gender	Size (mm)/ secretion	Ki67 (%)	Weiss score	Outcome/survival
C1	24F	105 x 100 x 58 Androgens	5%	3	Alive – 7 years
C2	55F	190 x 120 x 85 Cortisol	70%	8	Alive – 6 years
C3	46F	98 x 105 x 68 Cortisol	<5%	2	Alive – 7 year *
C4	76F	105 x 90 x 52 None	NA‡	NA	Died – 4 years**

Table 5.3 Details of four patients with adrenocortical carcinoma in the UK cohort
C1 and C2 received mitotane as part of their ACC treatment.
C3 and C4 did not and had very close clinical and radiological follow up.
**patient died from a different cancer, *patient was treated as ACC due to the size of her tumour. ‡ Haemorrhagic on histology

There was only a small number of ACC within our cohort as this is a very rare cancer, making further work and analysis more difficult. However, given the number of APAs and the recent interest in primary aldosteronism (PA) with growing evidence of aldosterone excess leading to increased cardiovascular and metabolic risks (Catena et al., 2008; Fallo et al., 2006, 2012; Milliez et al., 2005; Rossi et al., 2008) and end organ damage (Savard et al., 2013), the study went on to look more specifically at each individual APA and its genetic variant. Next generation sequencing has rapidly increased knowledge on genetic mutations causing familial PA with different phenotypes. The first mutation described was *KCNJ5* (Choi et al., 2011) and since, then many others have been described: *ATP1A1, ATP2B3, CACNA1D* and *CTNNB1* (Akerström et al., 2016; Azizan et al., 2013; Beuschlein et al., 2013; Fernandes-Rosa et al., 2015; Williams et al., 2014). The commonest mutation is *KCNJ5* at about 40% of APAs, 7%

of ATPase mutations (*ATP1A1* more than *ATP2B3*) and 9% have *CACNA1D* mutations (Monticone et al., 2014). *CTNNB1* had a prevalence of 5% in one study (Akerström et al., 2016).

5.7 Genetic mutations in APAs

In our cohort (Table 5.4) , *KCNJ5* was the commonest mutation at 48%, similar to published data and the ATPase mutations found in 12%, higher than published data (Williams et al., 2014). *KCNJ5* mutations were found more commonly in females (Table 5.5) and they are of zF type predominantly. There was no significant difference in age or aldosterone levels. An interesting observation was that half of the patients with *KCNJ5* mutations co-secretes aldosterone and cortisol.

	KCNJ5	*ATP1A1*	*ATP2B3*	*KCNJ5*/*ATP* negative
Male	G151R (G to C) 2 G151R (G to A) 1 L168R (T to G) 0 Total = 3	1	1	7
Female	G151R (G to C) 4 G151R (G to A) 2 L168R (T to G) 3 Total = 9	0	1	3
TOTAL	12	1	2	10

Table 5.4 Genetic mutations found the UK Aldosterone producing adenoma cohort
Aldosterone producing adenomas of 25 patients were sequenced for *KCNJ5, ATP1A1* and *ATP2B3* only.

Characteristics	*KCNJ5*	*ATP1A1*	*ATP2B3*	*KCNJ5/ATP* negative
Mean Age in years (range)	47.25 (27-70)	64	43 (34-52)	47.2 (37-68)
M:F	3:9	1:0	1:1	7:3
Mean Aldosterone pmol/L (range)	988.25 (405-1997)	782	1230 (490-1971)	1280.2 (406-3970)
Histology**	4 zF 1 zF/zR	zF/zR	1 zF 1zG/zF	5 zF 1 zF/zR 2 zR
Co-secretors*	6	0	0	0

Table 5.5 Summary of the characteristics of each genetic mutation group
*cortisol <50nmol/L after low dose dexamethasone suppression test (0.5mg dexamethasone six hourly for 48 hours)
**incomplete collection of histological phenotype of all the patients

5.8 Discussion

Adrenal adenomas such as APAs are relatively common compared to adrenocortical carcinomas (ACC). The prognoses of the two conditions differ immensely. APAs are potentially curable surgically but ACC tend to present late and often with metastases, with a 5 year survival rate of less than 10% (The American Association of Endocrine Surgeons – Patient education site). To date, there are no satisfactory treatments for ACC and survival rates have not improved over the decades. The options currently would be surgery, aiming for cure or for debulking. This is followed by mitotane (o,p-DDD) which inhibits side chain cleavage and 11 β-hydroxylase and hence adrenocortical steroidogenesis. It also acts by inducing hepatic clearance of cortisol. However, mitotane has many side effects which limits its use in many patients (Hogan et al., 1978; Huang and Fojo, 2008). If mitotane is tolerated, reaching therapeutic levels, then it contributes to recurrence free survival, though overall survival may

not be affected (Else et al., 2014). There are mixed reviews for the use of local radiotherapy to the adrenal bed, though the recent review by Else *et al.,* felt that adjuvant radiotherapy can prevent local recurrence and reduce morbidity caused by large recurrences (Else et al., 2014). Other newer therapies such as epidermal growth factor receptor (EGFR) inhibitors, anti-vascular growth factor antibodies and tyrosine kinase inhibitors have all had disappointing results on overall survival (Berruti et al., 2012; Cherradi, 2015; Kroiss et al., 2012; Quinkler et al., 2008). The identification of potential pathways that are involved in adrenal tumourigenesis would be a big step to understanding the pathogenesis of ACC and more targeted therapy in the future. The pathways investigated in this chapter were the Hh, Dlk1 and BMP pathways.

BMP2 and *BMP5* down regulation in ACC have already been shown (Johnsen et al., 2009). The reduction in *BMP2* expression in the ACC samples in this project, however, did not reach significance. This project has also shown that *BMP3b* may be overexpressed in ACC, though when this is looked at in more detail, only 2 out of the 4 ACCs showed an overexpression of *BMP3b*. The two that had the overexpression were ACCs of a less aggressive nature histologically and the patients are still alive. The down regulation of *BMP2* and the over expression of *BMP3b* seen, especially in Figure 3.6, which ties in well with our *in vitro* findings as the higher the *BMP3b* expression, the lower the expression of *BMP2* seen in patients C3 and C4. Whether there was a direct link between the BMPs in the ACC samples was not investigated, as this would have required manipulation of the cells through primary culture but BMPs are well known to have the potential to be either tumour suppressors or promoters in different cancers (Ehata et al., 2013). For example, *BMP2* is a tumour suppressor in diffuse type gastric carcinomas (Shirai et al., 2011), breast cancers (Ghosh-Choudhury et al., 2000) and renal cell carcinoma (Wang et al., 2012b) yet it can also stimulate cancer cell proliferation in other types of cancer, such as the prostate, in the absence of androgen presence (Ide et al., 1997). The widespread use of recombinant *BMP2* has also raised concerns on cancer development, again indicating that *BMP2* can also promote cancers (Carragee et al., 2013).

If BMPs were involved in ACC, this could also lead to potential therapeutic options in the future. Rodent studies with Bmp7 therapy have shown an improvement in renal disease in

diabetic rats (Wang et al., 2003) and other BMPs are being studied and used in bone regeneration (Krebsbach et al., 2000). BMP2 is already used in humans for spinal fusions since its approval by FDA in 2002 (Kleeman et al., 2001). However, complications of using this are now emerging, as many are being used 'off-license' and the FDA has issued warnings on its use (Epstein, 2013). This effect may have an impact on future developments of clinical application of BMPs. Using BMPs as gene therapy would require a fine balance as they are present in most organs and their function can vary vastly between organs (Wang et al., 2014). This project has shown that BMPs are present in the adrenal glands but expression levels have been variable in the different tissue samples. There was an overall suppression of *BMP3b* in APAs, except in one German sample and an increase in 2 of the 4 ACC samples from the UK cohort. The increase found in the less aggressive tumours could be interesting as it could be used as a good prognostic factor. However, the study of more ACCs will be needed in order to validate this theory.

There has been a surge in research into the pathogenesis of APAs. Up until 2010, there was only one genetic mutation associated with primary aldosteronism, a *CYP11B1/CYP11B2* chimeric gene, which results in type 1 familial primary aldosteronism (glucocorticoid remediable aldosteronism) and these patients are treated with glucocorticoids. There has now been a big shift into the genetics of APAs with many more candidate genes being discovered including the ones we have tested for, with others emerging. The cohort that has been genetically tested is small but the results concur with some of the findings in published data (Boulkroun et al., 2012): *KCNJ5* is the commonest mutation (48%) and is also more common in females. However, in this cohort, there was no difference in the age of presentation or the level of aldosterone. Reports also suggest a higher prevalence of *ATP1A1* (about 6%) compared to *ATP2B3* (2%) (Beuschlein et al., 2013) but so far we have identified more *ATP2B3* (2) mutations than *ATP1A1* (1) in our cohort. More samples and studies are needed in our cohort as we have not investigated *CACNA1D* mutations (Scholl et al., 2013) and *CTNNB1* (Akerström et al., 2016) yet.

The genetic mutations found so far, accounting for just over 50% of all APAs, mainly affect the cell membrane potential. *KCNJ5* acts on the inward rectifying potassium channel reducing

channel selectivity and increasing sodium permeability. The ATPase mutations act on the Na/K ATPase, which affects the stimulation of aldosterone release. *CACN1AD* encodes the L type calcium channel, which affects membrane potential via intracellular calcium regulation. *CTNNB1* is the only mutation so far not associated with ion selectivity but the Wnt/β-catenin pathway as *CTNNB1* is the β-catenin gene. This shows that of the undiscovered genes, they may not all be ion channel specific. The Wnt and BMP pathways can work synergistically together, such as in osteoblast differentiation, so BMPs may also play a role in APAs. In this project, BMPs were investigated in APA samples. *BMP6*, which induces aldosterone production in adrenocortical cell lines, was reduced in APAs, which was unexpected. It could be explained that *BMP6*, although it can induce aldosterone production, can only enhance Ang II and not potassium induced aldosterone production (Inagaki et al., 2006). Therefore, as many of the APAs are ion channel, hence potassium driven, hyperaldosteronism, *BMP6* may not be upregulated in some APAs. *BMP2* expression was lower than normal and *BMP3b* expression was similar to normal tissue in APAs. *BMP2* has been shown *in vitro* to exert a more zR phenotype and so would be unlikely to be involved in the development of APAs as these adenomas only rarely show some zR morphology. *BMP3b* would be predicted to have a higher expression as it supports a zG phenotype, and therefore aldosterone production, but it was expressed at very similar levels to normal tissue. Our data show that *BMP3b* is expressed in the adrenal capsule, and the expression levels may depend upon the amount of capsule present in the tissue sample obtained. It is still unclear why many of the APAs have a zF phenotype, although it is thought that cells in APAs have an intermediate phenotype in between zG and zF. It has been reported that *KCNJ5* mutations are more likely to have a zF phenotype and *ATP1A1* and *CACNA1D* mutations have a more zG phenotype (Azizan et al., 2013, 2012). Another reason for a zF phenotype is that Choi *et al.,* when they discovered *KCNJ5*, postulated that they caused hypertrophy of aldosterone secreting cells and this might give the cells a much 'fatter' appearance, resembling zF cells (Choi et al., 2011). These cells still express *CYP11B2* and another zG specific marker, *Dab2* (Disabled 2) (Boulkroun et al., 2011). It is likely that these cells are derived from zG and APAs form as they begin to transition into a zF phenotype (as per the centripetal theory) but their function remains that of zG. Therefore, *BMP3b* expression may be low as cells are already beginning to transition to the zF phenotype, as

shown in our *in vitro* gradient theory or that *BMP3b* is not involved in APA formation or maintenance of these tumour cells.

In conclusion, these results provide evidence of the presence of BMPs in the adrenal cortex but their expression levels were significantly different to each other. This could be explained by the theory that BMPs may not be involved in the pathogenesis of these tumours, they are suppressed by another mechanism that is driving the tumourigenesis or that the 'normal' tissues used in this project were in fact 'adjacent normal', where the difference in gene expression may not be truly representative of expression within a whole normal adrenal gland. However, in the ACC subgroup, the results were in agreement with our *in vitro* findings where an upregulation of *BMP3b* led to decreased *BMP2* expression.

Interesting outcomes from these experiments were firstly; the increased expression of *BMP3b* in ACC with a less aggressive histology and could potentially be looked at as a prognostic factor for ACCs. Secondly, the observation of increased co-secretion of aldosterone and cortisol in patients with a *KCNJ5* mutation would warrant further investigation.

6 General discussion

The adrenal gland is essential for life and understanding its differentiation, maintenance and zonation mechanism is vital to understand for health and in disease. Microarray results found *Bmp3b* expression to be exclusive to the outer cortex which includes the capsule and zG. Given its restricted expression, it was hypothesised that *Bmp3b* played a role in zonation by influencing zG differentiation from, and/or maintenance of, the progenitor/stem cells found beneath the adrenal capsule.

H295R cells were a useful model to investigate zonation, as the cells are able to express all the enzymes required for steroidogenesis and are able to produce aldosterone, cortisol and DHEA. This cell line can also be manipulated by prolonged treatment with Angiotensin II (to induce aldosterone production) and forskolin (to induce cortisol production). With this model, it was shown that BMP3b is present in adrenocortical cells, can upregulate *CYP11B2* and down regulate *CYP11B1, CYTB5* and *SULT2A1*. It also had a direct suppressive effect on *BMP2,* which is found most highly expressed in the inner cortex and promotes the zR cell phenotype, with these effects reversed on knocking down *BMP3b* with targeted siRNAs. Therefore, from the *in vitro* experiments, we propose that *BMP3b* acts to promote a zG phenotype but as cells move centripetally, the cells move down the concentration gradient away from the influence of BMP3b and are then under the influence of BMP2, which promotes a zF/zR phenotype. This model fits well with the centripetal theory of cell migration and that cells have the potential to de-differentiate as they migrate to take on a new phenotype (Freedman et al., 2013; Kim et al., 2009; Salmon and Zwemer, 1941; Wright et al., 1973).

Additional H295R studies would be useful to explore the interaction of BMP3b with other BMPs within the adrenocortical cells, such as BMP6, which plays a role in Ang II driven aldosterone production (Inagaki et al., 2006, 2007; Otani et al., 2010). The interplay of *BMP3b* with other known capsular/zG pathways would also be of interest. In other systems, *BMP* pathways work closely with *WNT* and *SHH* signalling pathways in development (Ding and Wang, 2017; Hu et al., 2005). *BMP* and *WNT* cross talk is already well established and they even share a common complex (Smad/β-catenin/Lef protein) that regulates shared target genes (Itasaki and Hoppler, 2010). *BMP4* has been shown to be dependent on β-catenin expression in colon cancer (Kim et al., 2002) and *BMP2/4* can regulate *Wnt8* (Hoppler and Moon, 1998).

Smad7, part of the *BMP* pathway, is also able to bind directly to β-catenin and is involved in prostate cancer (Edlund et al., 2005). Hence, it is highly likely that there is cross talk within the adrenal cortex that involves any combination of these pathways, with BMP3b involved, in part, in zG maintenance and differentiation. This would require further investigation in the future.

Bmp and *Hh* expression has been shown to co-localise in many areas, such as teeth, hair and gut (Bitgood and McMahon, 1995) and *Shh* can regulate *BMP2* in chick limb bud formation (Laufer et al., 1994). Vidal *et al.,* showed that *Rspo3* can initiate *Shh* activation and differentiation of steroidogenic cells through *Gli1* activation. We can extend this further and investigate if *Bmp3b* co-localises with *Rspo3* in the adrenal capsule, or if it contributes to or is independent of *Rspo3* to maintain a zG phenotype. This can be achieved on an *in vitro* level by looking at the expression of *Bmp3b* in the presence and absence of *Shh* and *Rspo3* and if *Bmp3b* expression is affected. The *in vivo* model can then be used to see if *BMP3b* is present in *Shh* or *Rspo3* KO models and if *Bmp3b* can reverse any of the effect of *Shh* and *Rspo3* KO models (Vidal et al., 2016).

Taking this hypothesis further in an *in vivo* model, the adrenal glands of the *Bmp3b* KO mouse was studied. *Bmp3b* KO mice are viable but their adrenal glands had not been studied (Zhao et al., 1999). The *Bmp3b* KO mice we studied was not histologically or morphologically different from wild type animals, indicating that adrenal development and differentiation does not solely rely on *Bmp3b* expression. This is supported by other published evidence of the importance of *Wnt, Shh* and *FGF* pathways in adrenal development and zonation (Guasti et al., 2010, 2013b; Vidal et al., 2016). Future work with BMP2 and BMP3b soaked Affigel beads to study the effect of *BMP2* and *BMP3b* will be useful to understand their specific sites of action by inserting the beads randomly into the adrenal cortex at different depths and on sacrifice, investigating the histology and expression of zone-specific markers around the beads. If BMP3b and BMP2 can only act in the subcapsular region and the zR, respectively, then only beads placed in these locations would be expected to demonstrate an effect on zonation. However, if their effect is not dependent upon the site of insertion, then BMP2 or BMP3b

released from the beads would cause changes within the specific zones, regardless of where the beads are inserted.

Studies using rodent models are straightforward in terms of accessibility and ethics. However, performing *in vivo* experiments requires an accumulation of skills in handling and performing surgery on these animals. During the course of this project we were unable to keep the Affigel beads in place in the adrenal gland for a period of 7 days in rat adrenal glands hence no results could be drawn from these experiments. Rodent data is not directly applicable to the human case, as although they do share some similarities structurally in terms of zonation, they differ in their steroidogenic output. Rodents lack *Cyp17* and are not able to produce cortisol or androgens. This could mean that the presence and expression of BMPs could differ between the two species. However, gathering initial data from rodents would help with future human studies.

During this project, *BMP2* and *BMP5* were genes of interest as they were shown to be downregulated in human adrenocortical carcinoma (Johnsen et al., 2009). This led the project to look at *BMP3b* in tumourigenesis. There was a downward trend in *BMP2* expression level in the ACC cohort, although this did not reach significance. Overall, *BMP3b* was upregulated in ACC but on closer inspection of individual patients, only half of the patients demonstrated this trend. The patients with increased *BMP3b* had less aggressive tumours on histology. In the German samples *BMP3b* was not upregulated in any of the samples, although no clinical data was available to correlate the aggressiveness of the tumours we received for investigation. Clearly more work needs to be done on ACCs to look for prognostic factors or potential pathways involved in driving ACC formation. However, progress has been limited by tissue collection in such a rare disease and collaboration between centres and countries is necessary to take such studies forward. Understanding the pathways involved in ACC will allow targeted therapies to be developed. Some therapies are already being used in clinical trials. *IGF2* is overexpressed in ACCs (Assié et al., 2010; Heaton et al., 2012; Soon et al., 2009) and an anti-IGF1R monoclonal antibody (Haluska et al., 2010) is currently used in clinical trials as lone therapy or in combination with mTOR inhibitor (Naing et al., 2013), though results have not

been promising. Therefore, for ACC treatment to be successful, we may need to target several pathways, and *BMP* may be one of these which is worth exploring in the future.

APAs are benign tumours of the adrenal cortex. Although the tumour itself is benign, the effects of aldosterone excess are not. Other than the effects of uncontrolled hypertension, hyperaldosteronism has independent effects on cardiovascular, renal and metabolic morbidity and mortality (Rossi et al., 2008). APAs are the commonest treatable cause of hypertension if diagnosed, but many go undiagnosed for many years, as it was previously thought to be a rare condition. The prevalence of APAs, with more readily available screening tests, is about 11% in all hypertensive patients and even higher in the resistant hypertension cohort (Rossi et al., 2006). Therefore, understanding the mechanism of hyperaldosteronism is important to offer treatment for those that cannot undergo surgery and those diagnosed with BAH, where surgery is not appropriate. In this project, *BMP3b* was downregulated in the UK cohort but appears upregulated in the German cohort, although we lacked German normal control samples for comparison. *BMP3b* is associated with zG phenotype and upregulates *CYP11B2*, hence aldosterone production, so it would be predicted that *BMP3b* would be overexpressed in APAs but this was not the case and *BMP3b* may not be involved in APA formation or maintenance. Within the last decade, genetics have superseded the diagnosis of APAs with the discovery of mutations within genes coding for ion channels (*KCNJ5, ATP1A1, ATP2B3* and *CACNA1D*) and most recently the *WNT* signalling pathway, *CNNTB1* (Akerström et al., 2016; Beuschlein et al., 2013; Choi et al., 2011; Scholl et al., 2013). This has increased our understanding of APAs with many studies attempting to correlate the genetics with clinical presentation and possibly treatment outcomes (Azizan et al., 2012; Kitamoto et al., 2015, 2016; Lenzini et al., 2015). So far, these mutations account for approximately 50-60% of APAs, therefore, the scope to discover more mutations associated with APAs and clinical correlation may be important as the genetics may dictate treatment and prognosis, such as in glucocorticoid remedial hyperaldosteronism, where a mutation in the chimeric *CYP11B1/CYP11B2* gene, is treated successfully with glucocorticoids (Lifton et al., 1992). Future studies of APAs would require detailed clinical information and a big cohort of patients to allow correlation with the genetic findings. There may be phenotypic differences in patients with APAs with and without a known genetic mutation. In addition, further work should be carried out in the *KCNJ5*

subgroup, with an unexpected increased incidence of aldosterone and cortisol co-secreting adenomas and to further characterise them.

In conclusion, this project has shown the presence of *Bmp3b* in the adrenal capsule as discovered in the rat microarray. It is potentially able to act locally to support zG differentiation and in a gradient to affect *Bmp2* expression and thus the differentiation into other zone specific phenotypes. *BMP3b* is likely to play a role in zonation, rather than being the main pathway, as the absence of *Bmp3b* is viable and the adrenals have not been shown to be severely affected. From the limited samples available it is tantalising to speculate that there is a role for *BMP3b* in tumourigenesis but this needs to be further studied using a much larger patient cohort. Ultimately, BMP3b may play a role in fine tuning adrenocortical cell differentiation but the lack of a mouse phenotype suggests it is unlikely to be essential in adrenal differentiation and zonation. There were many limitations to this project and further investigations with adequate controls will be needed in the future to further contribute to the unravelling of the many different pathways that could interact and determine adrenocortical zonation.

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8 APPENDIX

List of reagents used and Suppliers

Abcam:

BMP2 antibody

Side chain cleavage antibody

Ambion:

10x turbo buffer

Glycogen

siBMP2

siBMP3b

Abnova:

Recombinant BMP3b

BioSepra:

Ultroser G

Demeditec Diagnostics

Aldosterone ELISA kit

Cortisol ELISA kit

DHEAS ELISA kit

Gibco, Paisley, UK:

F12 nutrient media (Ham)

Invitrogen:

Goat anti mouse antibody

Goat anti rabbit antibody

Lipofectamine 2000

Optimem I

Jackson ImmunoResearch:

Donkey anti goat antibody

Donkey anti rabbit antibody

Lamb Laboratories

OCT medium -

New England Biolabs, MA, US

S6 RNA polymerase (M0207S)

T7 RNA polymerase (M0251S)

Taq DNA Polymerase wit standard Taq buffer (M0273S

Promega:

2x rapid ligation buffer

5x reverse transciptase

10x restriction buffer –

dATP, dCTP, dGTP, dTTP

E.Coli competent cells JM109

Isopropyl-β-d-thiogalactopyranoside (IPTG)

M-MLV Reverse Transcriptase (M1701)

PGEMTeasy system 1 (A1360)

Random primers (C118A)

RNase Inhibitor

T4 DNA ligase

X-Gal (V3941)

Qiagen:

DNase I, RNase-Free

RNase-Free water

RNeasy kit

Roche:

0.5% Blocking Reagent

Anti-DIG alkaline phosphatase-Fab fragments

DIG RNA Labellling Mix 10x conc

NBT/BCIP

Santa Cruz Biotechnology, INC:

BMP2 antibody for immunofluorescence (BMP2 (N-14): sc-6895)

Sigma-Aldrich:

8-Hydroxyquinoline

Acetic acid

Agar powder

Agarose powder

Angiotensin II (human) (A9525)

Bovine serum albumin (BSA)

Denhardt's solution

Denatured salmon sperm DNA

Deoxyribonuclease I

Diethyl pyrocarbonate (DEPC)

Dimethyl sulfoxide (DMSO)

DPX mounting medium

Dulbecco's Modified Eagle's Medium (DMEM)

Ethylenediaminetetraacetic acid (EDTA)

Ethidium bromide

Fetal bovine serum (FBS)

Forskolin

Insulin-Transferrin-Selenium (ITS) (100 x)

LB Broth

Orange G

Phosphate buffered saline (PBS)

Penicillin/Streptomycin

Phenol

SOC medium

Sodium acetate

TESPA (3-triethoxysilylpropylamine)

Tris base

Tris hydrochloride

Thermo Fisher Scientific:

GelRed

GeneRuler DNA Ladder mix

Shenandoah Biotechnology, inc:

Recombinant Human BMP2

Vectorlabs:

ABC reagents (Avidin + Biotinylated horseradish peroxidise macromolecular Complex)

Avidin

Biotin

Levisimole

Generous gift from Prof Gomez-Sanchez:

CYP11B1 antibody

CYP11B2 antibody

Generous gift from Prof Gavin Vinson:

Pref-1 (Dlk1) antibody

Abcam	330 Cambridge Science Park, Cambridge CB4 0FL
Abnova	Boxbergring 107, 69126 Heidelberg, Germany
Ambion	Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF
Biosepra	Clarendon House, 125 Shenley Rd, Borehamwood, Hertfordshire WD6 1AG
Demeditec Diagnostics	Lise-Meitner-Str. 2 24145 Kiel, Germany
Invitrogen	Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF
Jackson ImmunoResearch	Unit 7, Acorn Business Centre, Oaks Drive, Newmarket, Suffolk CB8 7SY
Kapa Biosystems	Anachem House, 1 & 2 Titan Court, Laporte Way, Bedfordshire LU4 8EF
Lamb Laboratories	Fisher Scientific, Bishop Meadow Road, Loughborough LE11 5RG
New England Biolabs	75-77 Knowl Piece, Hitchin SG4 0TY
Promega	Delta House, Southampton Science Park, Southampton SO16 7NS
Qiagen	Fleming Way, Crawley, West Sussex RH10 9NQ
Roche	Roche Diagnostics Limited, Charles Avenue, Burgess Hill, West Sussex, RH15 9RY
Shenandoah Biotechnology	Shenandoah Biotechnology, Inc., 101 Camars Drive, Warwick, PA USA 18974
Sigma-Aldrich	The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT
Thermo Fisher Scientific	Stafford House, 1 Boundary Park, Hemel Hempstead HP2 7GE
Vector Laboratories	3, Accent Park, Bakewell Road, Orton Southgate, Peterborough PE2 6XS